CLINICAL ASPECTS OF T-CELL AGEING IN END-STAGE RENAL DISEASE PATIENTS

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CLINICAL ASPECTS OF T-CELL AGEING IN END-STAGE RENAL DISEASE PATIENTS

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"Science is the most reliable guide in life"

M.K. Atatürk

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GENERAL INTRODUCTION
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INTRODUCTION

Renal disease and the immune system

Chronic kidney disease (CKD) is a global problem with a worldwide prevalence of approximately 13% (1). The major causes of CKD are hypertension and diabetes (2). According to the 'Kidney Disease, Improving Global Outcomes' (KDIGO) guidelines, CKD is defined as a glomerular filtration rate (GFR) below 60 ml/min/1.73m^2 for ≥3 months (3). Based upon the GFR, CKD is divided into 5 stages (3). When the GFR drops <15 ml/min/1.73m^2, also known as end-stage renal disease (ESRD), this is defined as renal failure (3). Patients with ESRD need renal replacement therapy (RRT) in the form of dialysis or renal transplantation, with the latter being the best option for RRT because it is associated with a better survival and quality of life compared with dialysis (4-6). Renal failure is associated with an impaired immune system and ESRD patients are known to be more susceptible to infections and show lower vaccination responses to vaccinations when compared with healthy individuals (7-10). Next to this, recent studies show that patients with ESRD show a prematurely aged T-cell immune system when compared with healthy individuals (11-13).

Ageing of the circulatory T-cell compartment

With increasing age, there are several changes that occur in the (T-cell) immune system. Firstly, hematopoietic stem cells shift towards the myeloid progenitor line, which causes a decline in the lymphoid progenitor cells (14). Next to this, the thymus involutes, which leads to a decline in functional tissue and a decline in newly formed naive T cells (15). These newly formed naive T cells, also called recent thymic emigrants (RTEs), can be recognized by their expression of CD31 on their surface (16, 17). RTEs are also high in T-cell receptor excision circle (TREC) content (16, 18). TRECs arise during T-cell receptor (TCR) rearrangement in the thymus. During TCR rearrangement in the thymus, parts of DNA in the TCR loci will be deleted and circularized into these molecules (18). An interesting characteristic of these TRECs are that they are passed through to only one daughter cell during proliferation as they are located outside the nucleus and do not replicate during cell division (19) (Figure 1). Thus, dilution of the TREC content is informative about the proliferative history of T cells. This means that the frequency of RTEs and the TREC content give information about the thymic output and can be used as T-cell ageing parameters.

Furthermore, ageing also causes an increase in differentiation and proliferation of circulatory T cells. This will lead to phenotypical changes in T cells (Figure 2). Naive T cells will differentiate into memory T cells and these memory T cells will comprise the majority of the peripheral T-cell pool in the elderly (20, 21). This will eventually lead to the loss of the homing receptor CCR7, loss of CD27 and upregulation of PD-1 and CD57 (22-26). Next to this, it is also associated with the loss of the co-stimulatory molecule CD28 on the surface of (highly differentiated) memory T cells (27). This co-stimulatory molecule

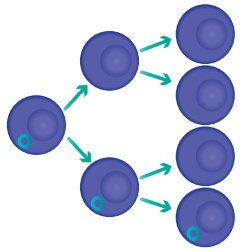

Figure 1. The TRECs (indicated with the blue circles) are only passed through to one daughter cell after division.

binds to CD80/86 which is needed for a proper T-cell activation when the TCR binds to a major histocompatibility (MHC) peptide complex (28). When a T cell only binds to an antigen presented by an MHC molecule without the simultaneous stimulation of CD28, it will go into apoptosis or become anergic (28). These highly differentiated memory T cells, also defined as $CD28^{null}$ memory T cells, have a high cytotoxic potential and can already become activated by the recognition of an antigen without the need for co-stimulation (27, 29). These T cells are present within both the $CD4^+$ and $CD8^+$ T-cell populations (23, 27, 29, 30). The $CD4^+CD28^{null}$ T cells resemble $CD8^+CD28^{null}$ T cells with regard to their properties. They are able to produce proinflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and have intracellular stores of granzyme B and perforin (23, 29-32). Circulatory $CD4^+CD28^{null}$ T cells have been associated with various clinical outcomes. They are associated with unstable angina, atherosclerotic vascular events and risk for the development of (co-stimulatory blockade resistant) rejection after renal transplantation (29, 31, 33-36). Therefore, the presence of these cells plays an important role with regard to clinical outcomes in ageing individuals.

Special attention needs to be given to infection with cytomegalovirus (CMV) with regard to the increased differentiation of T cells. CMV is a member of the herpesviridae family and has the largest genome of all the herpesviruses (37). The prevalence increases with age, which can be >60% among people who are over 40 years old (38). An infection

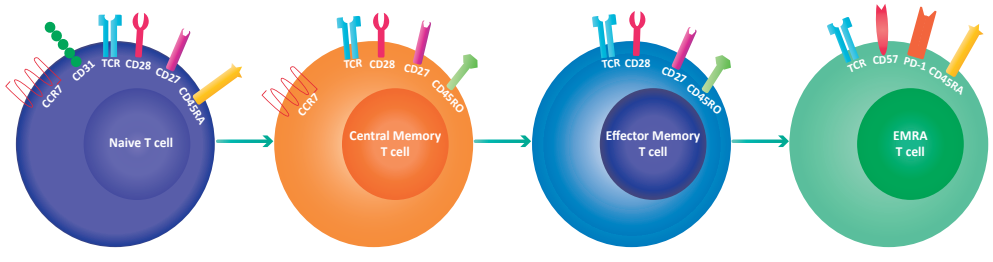

Figure 2. Differentiation of T cells leads to loss of markers such as CCR7, CD28, CD27 and the upregulation of CD57 and PD-1.

is also influenced by ethnicity and wealth (38, 39). For example, the prevalence is higher in Hispanics and people with a low socioeconomic status (38, 39). An acute infection is usually asymptomatic, but might also be accompanied by symptoms that resemble infectious mononucleosis (40). After an acute infection, the virus remains latent within the body (41). CMV can lead to the appearance of late differentiated memory T cells, especially of memory T cells that lack the co-stimulatory molecule CD28 (21, 42, 43). Next to this, it has also been associated with an inversion of the CD4:CD8 ratio, lower relative telomere length of $CD8^+$ T cells, and a decline in RTEs and TREC content (21, 44). Thus, CMV causes significant changes in the circulatory T-cell system that mimics ageing.

In addition to the increased differentiation, the increase in proliferation will also affect the telomere length of the circulatory T cells, leading to telomere attrition (45, 46) (Figure 3).

Ageing of T cells within lymph nodes

Lymph nodes are oval shaped structures that are widely distributed throughout the human body and are connected with each other by lymphatic vessels. They are responsible for the circulation of lymph, which is a clear, plasma-like fluid that consists of leukocytes, bacteria, proteins and cellular waste products. Lymph enters the lymph node via afferent lymphatic vessels and leaves the lymph node via efferent lymphatic vessels. A lymph node is covered by a fibrous capsule. The tissue itself is further divided into a cortex and a medulla. The outer part of the cortex consists of lymphoid follicles, which mainly contain B cells. The deeper part of the cortex mainly consists of T cells and dendritic cells. The medulla consists of plasma cells that secrete antibodies and macrophages (47, 48).

Lymph nodes are an important part of the immune system, as immune responses are initiated within lymph nodes. B cells will form germinal centers within the lymph node after recognizing an antigen via interaction with follicular dendritic cells. Dendritic cells in the deeper part of the cortex, will present antigens to naive T cells, which then will proliferate and differentiate into effector cells. Effector T cells will then leave the lymph node to migrate to the site of infection (47, 48). These responses can lead to an enlargement of the lymph node, which can be observed by the human eye.

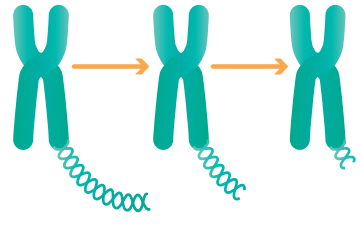

Figure 3. Every division leads to attrition of telomeres, which are located at each end of the chromatid.

Until now, little is known about how ageing-related changes in circulatory T cells relate to changes within the lymph node (49, 50). Next to that, whether the circulatory changes in ESRD patients also occur within lymph nodes is unknown. Thus, an in depth analysis of these changes within lymph nodes is also needed for a more comprehensive understanding of the ageing T-cell immune system in ESRD patients.

THE AIM AND OUTLINE OF THIS THESIS

Patients need to use immunosuppressive agents after renal transplantation which has disadvantages. Immunosuppressive drugs will reduce the risk for rejection, but on the other hand will increase the risk for diabetes, hypertension, infections, and they can even be harmful for the renal allograft because of their nephrotoxicity (51, 52). Since ESRD patients show a prematurely aged, circulatory T-cell immune system (11, 12), the use of these agents will impair their immune system even more. Therefore, definition of predictive T-cell parameters with regard to the development of rejection and infection is needed for a more personalized immunosuppression. Next to this, the ageing-related changes in circulatory T cells need to be compared with the changes in lymph nodes, since immune responses are initiated within lymph nodes. Thus, **the objective of this thesis** is, to evaluate the predictive value of T-cell ageing parameters and clinical outcomes such as the development of rejection and infection, and also to compare the changes in circulatory T cells with changes in the lymph nodes.

Chapter 1 will introduce the premature ageing process in ESRD patients and the different T-cell ageing parameters that can be assessed from the blood and lymph nodes.

Chapter 2 will discuss the relationship between circulatory T-cell ageing parameters assessed prior to renal transplantation and the development of acute allograft rejection within three months after renal transplantation.

Chapter 3 will describe the alloreactive potential of $CD4^{+}CD28^{null}$ T cells, as these cells were associated with a reduced risk for rejection after renal transplantation.

Chapter 4 will assess the relationship between circulatory T-cell ageing parameters and the development of opportunistic and serious infections after renal transplantation.

Chapter 5 will describe how ageing-related changes in circulatory T cells relate to changes in the T-cell composition of lymph nodes.

Chapter 6 will evaluate the relationship between the development of early acute rejection after renal transplantation and the T-cell composition of the lymph node prior to renal transplantation. Next to this the alloreactivity of T cells isolated from lymph nodes and peripheral blood were also evaluated and compared.

Chapter 7 will summarize and discuss all the obtained results

Chapter 8 will show a Dutch summary of the acquired results

Chapter 2

LOSS OF CD28 ON PERIPHERAL T CELLS DECREASES THE RISK FOR EARLY ACUTE REJECTION AFTER KIDNEY TRANSPLANTATION

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ABSTRACT

Background

End-stage renal disease patients have a dysfunctional, prematurely aged peripheral T-cell system. Here we hypothesized that the degree of premature T-cell ageing before kidney transplantation predicts the risk for early acute allograft rejection (EAR).

Methods

222 living donor kidney transplant recipients were prospectively analyzed. EAR was defined as biopsy proven acute allograft rejection within 3 months after kidney transplantation. The differentiation status of circulating T cells, the relative telomere length and the number of $CD31^+$ naive T cells were determined as T-cell ageing parameters.

Results

Of the 222 patients analyzed, 30 (14%) developed an EAR. The donor age and the historical panel reactive antibody score were significantly higher (p=0.024 and p=0.039 respectively) and the number of related donor kidney transplantation was significantly lower (p=0.018) in the EAR group. EAR-patients showed lower $CD4^+CD28^{null}$ T-cell numbers ($p<0.01$) and the same trend was observed for $CD8^+CD28^{null}$ T-cell numbers (p=0.08). No differences regarding the other ageing parameters were found. A multivariate Cox regression analysis showed that higher $CD4^+CD28^{null}$ T-cell numbers were associated with a lower risk for EAR (HR: 0.65, p=0.028). In vitro, a significant lower percentage of alloreactive T cells was observed within $CD28^{null}$ T cells ($p<0.001$).

Conclusion

Immunological ageing-related expansion of highly differentiated $CD28^{null}$ T cells is associated with a lower risk for EAR.

INTRODUCTION

Loss of renal function leads to retention of uremic molecules and cytokines, which creates oxidative stress and inflammation (53). The resulting pro-inflammatory uremic environment underlies the dysfunctional T-cell immunity of end-stage renal disease (ESRD) patients (54). The major changes in the peripheral T-cell composition are T-lymphopenia, increased T-cell differentiation and loss of telomere length, the latter indicating a history of enhanced T-cell replication (55).

The T-lymphopenia is largely due to a loss of naive (antigen-inexperienced) T cells, which show signs of increased activation and are more prone to apoptosis (55). This loss of circulating naive T cells runs in parallel with a decrease in newly formed naive T cells, known as recent thymic emigrants (RTEs, indicating a premature involution of the thymus). In combination with an expanded, more differentiated memory T-cell compartment, this leads to a relatively large decrease in the percentage of circulating naive T cells (55, 56). The highly differentiated memory T cells are characterized by a loss of the co-stimulatory molecule CD28, making them less dependent on co-stimulation to become activated (27). Moreover, these cells are known to have a reduced telomere length due to their numerous cell divisions (30, 55, 57).

The uremia-associated changes in the composition of the peripheral T-cell compartment resemble the physiological changes in the ageing immune system of elderly healthy individuals, (13, 20, 58) which leads to the concept of ESRD-related premature immunological ageing. This was confirmed when a combined analysis of the thymic output, differentiation status and the telomere length of T cells in ESRD patients was performed and the results were compared to healthy individuals over a wide age range (55). A consistent pattern of premature immunological ageing was observed with a discrepancy of 15-20 years between the immunological age of T cells of ESRD patients compared to their chronological age (12, 55). This prematurely aged T-cell system of ESRD patients offers at least a partial explanation for the increased susceptibility to infections (7), reduced vaccination response (8-10, 59), increased prevalence of malignancies (60, 61) and may also be a non-classical risk factor for cardiovascular diseases (62-65).

A prematurely aged T-cell system leading to impaired T-cell immunity may also reduce the risk for acute rejection after kidney transplantation, but this has not been systematically studied. In addition, most studies that have assessed the circulating T-cell compartment in relation to acute rejection have only demonstrated percentages of cells (66, 67). This can lead to erroneous conclusions given the complex changes in all T-cell subsets and for example expansion of memory T cells may be interpreted as a reduction in the number of naive T cells and vice versa.

In this study, we hypothesized that the degree of premature T-cell ageing, based on the absolute number of differentiated T cells, thymic output and telomere length, prior to kidney transplantation (KT) is associated with the risk for early acute allograft rejection

(EAR) in kidney transplant recipients. Based upon our analyses we observed that T-cell differentiation status was associated with the risk for EAR after KT.

MATERIALS AND METHODS

Study population

All patients participated in a randomized-controlled clinical trial with the primary aim to study the efficacy of a genotype-based approach to tacrolimus dosing (Dutch trial registry number NTR 2226; http://www.trialregister.nl/trialreg/index.asp). All patients gave written informed consent to participate in the clinical trial, as well as for the sub-study, which is presented here. None of the transplant donors were from a vulnerable population and all donors or next of kin provided written informed consent that was freely given. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2010-080, EudraCT 2010-018917-30). This study was conducted in accordance with the Declaration of Helsinki.

All patients undergoing a living-donor KT in the period from 1 November 2010 to 1 October 2013 were considered for participation in this study. This study included all ESRD patients with various causes of chronic kidney disease (CKD) (Table 1). Patients were excluded if they were younger than 18 years and if they received immunosuppressive medication (except for glucocorticoids) within 28 days prior to transplantation.

All patients received induction therapy with basiliximab (20 mg i.v. on day 0 and day 4), tacrolimus (aiming for predose concentrations of 10-15 ng/mL in weeks 1-2, 8-12 ng/mL in weeks 3-4, and 5-10 ng/mL, thereafter), mycophenolate mofetil (starting dose of 1 g b.i.d., aiming for predose concentrations of 1.5 – 3.0 mg/L), and glucocorticoids. All patients received 50 mg prednisolone b.i.d. intravenously on days 0-3. Thereafter, 20 mg oral prednisolone was started and subsequently tapered to 5 mg at month 3.

We determined clinical variables such as age at time of transplantation, gender, CMV-seropositivity, anti-CMV IgG titer, human leukocyte antigen (HLA) class I and class II mismatches, current and historical panel reactive antibody (PRA) score, warm ischemia time (WIT), number of previous kidney transplantations, preemptive KT (defined as receiving a kidney before the start of renal replacement therapy (RRT)) and related KT (defined as receiving a kidney from a genetically related donor) (Table 1). The HLA-typing was assessed according to the international standards (American Society for Histocompatibility and Immunogenetics/the European Federation for Immunogenetics) using serologic and DNA-based techniques. The PRAs were determined at the laboratory of the blood bank in Leiden, the Netherlands. In all transplantations the complement dependent cross match was negative, but flowcytometry based cross matches were not performed.

We defined EAR as the development of biopsy-proven acute allograft rejection according to the Banff criteria (68) within 3 months after KT.

PBMCs isolation

By using Ficoll-Paque Plus (GE healthcare, Uppsala, Sweden), peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples drawn from KT-recipients the day before KT. The isolated PBMCs were stored at –150°C with a minimum amount of 10×10^6 cells per vial for further experiments.

Absolute numbers of $CD4^+$ and $CD8^+$ T cells and T-cell differentiation status by FACS analysis

To determine the absolute numbers of the different lymphocyte populations from blood, a Trucount staining was done. In this protocol, 20 µl of the 6-color TBNK reagent (BD Multitest™, BD, Erembodegem, Belgium) was used in combination with a BD Trucount™ tube (BD) and 50 µl of EDTA blood. This tube contains a number of beads (i.e. bead count; lot-specific) and enables calculation of absolute numbers of cells per µl of blood. The 6-color TBNK reagent contains phycoerythrin (PE)-labeled anti-CD45, AmCyan-labeled anti-CD19, PE-Cy7-labeled anti-CD3, Peridinin chlorophyll (PerCP)-labeled anti-CD4, fluorescein isothiocyanate (FITC)-labeled anti-CD8 and allophycocyanin-Cy7 (APC-Cy7)-labeled anti-CD16/CD56.

In addition, a whole blood staining was performed to determine the T-cell differentiation status (55, 69). Briefly, whole blood was stained with AmCyan-labeled anti-CD3 (BD) in combination with pacific blue (PB)-labeled anti-CD4 (BD) and APC-Cy7-labeled anti-CD8 (BD). T cells were defined as $CD4^+$ or $CD8^+$ and further defined into four different subsets based on their expression of CCR7 and CD45RO after staining using FITC-labeled anti-CCR7 (R&D systems, Uithoorn, The Netherlands) and APC-labeled anti-CD45RO (BD). Naive T cells were identified as $CCR7^+$ and $CD45RO^-$, central memory (CM) cells as $CCR7^+$ and $CD45RO^+$, effector memory (EM) cells as $CCR7^-$ and $CD45RO^+$ and the highly differentiated effector memory $CD45RA^+$ (EMRA) cells as $CCR7^-$ and $CD45RO^-$. T-cell differentiation is associated with loss of CD28 expression on the cell surface. Numbers of $CD28^-$ (or $CD28^{null}$) T cells within the T-cell subsets were determined by staining with PerCP-Cy5.5-labeled anti-CD28 (BD). Recent thymic emigrants (RTEs) were identified by the expression of CD31 within the naive T-cell pool upon staining with PE-labeled anti-CD31 (Biolegend, Europe BV, Uithoorn, the Netherlands). Samples were measured at the FACSCanto II (BD) acquiring at least $5x10^4$ lymphocytes and analyzed using FACS Diva software version 6.1.2 (BD) (55, 69).

Telomere Length Assay

Flow fluorescent *in situ* hybridization (flow-FISH) was performed to determine the relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells. PBMCs were isolated and stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or CD8-biotin (Biolegend) followed by staining with streptavidin-Cy5 (Biolegend). The PBMCs were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and by using

the telomere FITC-labeled PNA-kit (DakoCytomation, Heverle, Belgium) the telomere length was determined. The sub cell-line 1301 of CCRF-CEM (human T-cell leukemia, Sigma-Aldrich, Zwijndrecht, the Netherlands, ECACC catalogue number 85112105) known for its long telomeres, served as an internal positive control. After acquisition of the samples on the FACSCanto II (BD) and analysis using FACS Diva software version 6.1.2 (BD), the RTL of the $CD4^+$ and $CD8^+$ T cells was calculated through the next formula (69, 70):

$$RTL = \frac{(\text{median FL1 sample cells with probe} - \text{median FL1 sample cells without probe}) \times DNA\ index\ of\ control\ (=2) cells}{(\text{median FL1 control cells with probe} - \text{median FL1 control cells without probe}) \times DNA\ index\ of\ sample\ (=1) cells} \times 100$$

Cytokine producing alloantigen-stimulated T cells

To determine frequencies of alloantigen-specific (cytokine-producing) T cells prior to transplantation, we used the CD137 multi-parameter flowcytometric assay as published recently (71). PBMCs of a kidney transplant recipient were stimulated in presence of co-stimulation (αCD49d, 1 μg/mL; BD) with or without T cell-depleted (>98% pure) donor PBMCs at a $1(5x10^6)$:$1(5x10^6)$ ratio for 24 hours of which the last 12 hours were in presence of Brefeldin A (Golgiplug, BD) and monensin (Golgistop, BD). A distinction between donor and recipient's cells is enabled by depleting the donor PBMC for $CD3^+$ T cells, i.e. $CD3^+$ T cells are derived from the recipient. Subsequently, the cell surface was stained using AmCyan-labeled anti-CD3 (BD), APC-Cy7-labeled anti-CD8 (BD) and PerCP-Cy5.5-labeled anti-CD28 (BD) in order to visualize where the cytokine producing T cells are located. Following fixation and permeabilization, CD137 and cytokines were stained intracellular using APC-labeled anti-CD137 (BD), PE-labeled anti-interferon (IFN)-γ (BD) and FITC-labeled anti-interleukin (IL)-2 (BD). At least $0.5\text{-}1\times10^6$ viable $CD3^+$ T cells were acquired on the FACS Canto II. Alloantigen-specific cytokine producing T cells were corrected for the cytokine signal observed in the absence of donor T-cell-depleted PBMC stimulation. Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD).

Statistical analysis

All variables are presented as medians with interquartile ranges. The difference between continuous variables was analyzed with the Mann–Whitney U test. The difference between categorical variables was analyzed either with the Pearson's chi-squared test or with the Fisher's exact test depending on the expected values in any of the cells of a contingency table. The latter was used when the expected values were lower than 5 in any of the cells. For the assessment of an association between clinical/immunological variables and the presence of EAR, the Cox proportional hazards model was used. Next to this, a Kaplan-Meier curve was created for the assessment of EAR free survival rate stratified for one of the T-cell ageing parameters. The significance level (p-value) was two-tailed and 0.05 was used for all analyses. Statistical analyses were performed using SPSS® version 21.0 for Windows® (SPSS Inc., IL, USA). T-cell subset graphs were created using GraphPad Prism 5 (CA, USA).

RESULTS

Patient characteristics

We enrolled 222 consecutive patients who received a kidney transplant from a living donor. Patient characteristics are shown in Table 1. Of the 222 patients analyzed, 30 (14%) had an EAR. The majority of the rejections were classified as grade II (67%), 30% were classified as grade I and only one rejection was classified as grade III. The median age of the patients was 57 years and the median donor age was 53 years. The donors were significantly older in the EAR group with a median age of 58 years compared to a median age of 52 years in the no rejection group (p=0.024). The majority of the patients (92%) received a donor kidney for the first time, 14 patients (6%) for the second time and three patients (1%) for the third time. Of the 30 patients who developed EAR, 11 (37%) received T-cell depletion therapy consisting of either alemtuzumab subcutaneously or rabbit anti-thymocyte globulin (rATG) intravenously. Four patients had a transplantectomy within the first 3 months after transplantation. Two of these patients had a therapy-resistant cellular acute rejection within the first week after KT. The other two patients were in the no rejection group and the graft was removed due to vascular problems that occurred during the transplantation procedure.

The historical PRA score was significantly higher in the EAR group compared with the no rejection group (p=0.039). The relative number of genetically related KT was significantly lower in the EAR group (20% vs 43%, p=0.018). Other potential risk factors for acute rejection, like number of HLA mismatches or previous kidney transplantation, did not associate with EAR in this patient group.

Patients with EAR have a lower number of $CD4^+CD28^{null}$ T cells prior to KT

The $CD4^+$ and $CD8^+$ T-cell differentiation status of both patient groups was determined prior to KT (Figure 1). In S1 Figure, typical examples of the gating strategy are depicted for the flowcytometric analysis of the $CD4^+$ and $CD8^+$ T-cell population, respectively. No significant differences were found in the number of $CD4^+$ T cells (Figure 1A) between the EAR group and no rejection group. Moreover, the number of naive, memory, CM and EM (Figure 1B-1E) was not significantly different. Interestingly, compared to the no rejection group, the EAR group had significant lower number of $CD28^{null}$ T cells within the $CD4^+$ T-compartment (21 cells/μl vs. 7 cells/μl, p<0.01, Figure 1F).

The total number of $CD8^+$ was not significantly different between the two patient groups (Figure 2A). The number of naive, memory and CM, EM and EMRA (Figure 2B-2F) $CD8^+$ T cells did also not show any significant differences between the two groups. Furthermore, the EAR group tended to have a lower number of $CD28^{null}$ $CD8^+$ T cells (p=0.08, Figure 1G) compared to the no rejection group.

Table 1. Patient characteristics

KT Patients (n=222)	No Rejection (n=92) (86%)	Early Rejection (n=30) (14%)	P
Age recipient	57 (46 – 64)	55 (47 – 63)	0.60
Age Donor	52 (40 – 62)	58 (50 – 65)	**0.024**
Male gender recipient	118 (61%)	14 (63%)	0.84
Male gender donor	94 (49%)	13 (43%)	0.57
CMV-seropositivity recipient	118 (61%)	15 (50%)	0.23
CMV-serostatus donor/recipient			
-/-	42 (22%)	10 (33%)	0.17
-/+	38 (20%)	5 (17%)	0.69
+/-	32 (17%)	5 (17%)	1.00
+/+	80 (42%)	10 (33%)	0.39
Anti-CMV IgG titer recipient (AU/mL)	65 (42 – 105)	58 (47 – 86)	0.75
Mismatch HLA class I	2 (2 – 3)	3 (2 – 3)	0.15
Mismatch HLA class II	1 (1 – 2)	1 (1 – 2)	0.31
Mismatch HLA class I and II	4 (3 – 5)	4 (3 – 5)	0.11
PRA current (%)	0 (0 – 4)	0 (0 – 4)	0.52
PRA historic (%)	4 (0 – 4)	4 (0 – 29)	**0.039**
Amount of KT	1 (1 – 1)	1 (1 – 1)	0.63
Warm ischemia time	20 (16 – 24)	21 (16 – 25)	0.69
Cause of CKD			
Nephrosclerosis/atherosclerosis/hypertension	44 (23%)	7 (23%)	0.96
Primary glomerulopathies	26 (14%)	4 (13%)	1.00
Diabetes	41 (21%)	2 (7%)	0.06
Urinary tract infections/stones	5 (3%)	1 (3%)	0.59
Reflux nephropathy	9 (5%)	1 (0%)	1.00
Polycystic Kidney Disease	33 (17%)	7 (23%)	0.42
Other	26 (14%)	5 (17%)	0.58
Unknown	8 (4%)	3 (10%)	0.17
Pre-emptive KT	75 (39%)	17 (57%)	0.07
Genetically-related KT	82 (43%)	6 (20%)	**0.018**
Acute rejection type			
Cellular rejection		25 (83%)	
Antibody-mediated rejection		1 (3%)	
Mixed rejection		4 (13%)	

Data are presented as medians (interquartile range).

Even though we mainly focused on absolute cell numbers, we also analyzed percentages of the different T cell subsets to provide a better comparison with previous studies. These analyses showed similar findings (S1 Table). Again, significant higher percentages of $CD4^+CD28^{null}$ T cells were seen in the no rejection group (p=0.011). Next to this, (a tendency for) higher percentages of $CD4^+$ and $CD8^+$ CM T cells were seen in the EAR group (p=0.055 and p=0.005 respectively).

No differences in relative telomere length and RTEs between the EAR group and no rejection group prior to KT

As a marker for the proliferative history, the RTL of the $CD4^+$ and $CD8^+$ T cells was determined. For both T-cell subsets no significant differences were found between the EAR group and the no rejection group regarding the RTL (Table 2).

The number and percentages of RTEs were identified by the expression of CD31 within the naive T-cell pool. No significant differences for $CD4^+$ or $CD8^+$ T cells were found between the two groups prior to KT (Table 2).

Donor age, historical PRA, a related kidney donation and absolute numbers of $CD4^+CD28^{null}$ T cells are related with the risk for EAR

The results of the univariate Cox regression analysis of the patient characteristics are presented in Table 3. This analysis showed that receiving an older donor kidney was associated with a higher risk for EAR (HR: 1.41, p=0.011). Besides this, a higher historical PRA score was also associated with a higher risk for EAR (HR:1.11, p=0.001). Receiving a donor kidney from a relative reduced the risk for EAR (HR: 0.36, p=0.025).

The univariate Cox regression analysis of the T-cell subsets showed that a higher number of absolute $CD4^+CD28^{null}$ T cells, i.e. having a more differentiated $CD4^+$ T-cell compartment, was associated with a lower risk for EAR (HR: 0.65, p=0.025). In contrast, the number of $CD8^+CD28^{null}$ T cells was not associated with the risk for EAR (HR: 0.98, p=0.420). Furthermore, higher percentages of $CD4^+CD28^{null}$ T cells were also associated with a lower risk for rejection (HR: 0.92; p=0.047). However, higher percentages of $CD8^+$ CM T cells, representative of having a less differentiated memory compartment, were associated with a higher risk for EAR (HR: 1.05; p=0.035).

A multivariate Cox regression analysis was performed with the three aforementioned clinical characteristics (i.e. donor age, historical PRA and a related kidney donation) as covariates (Table 4). In accordance with the univariate analysis, a higher absolute number of $CD4^+CD28^{null}$ T cells was associated with a lower risk for EAR (HR: 0.65, p=0.028). Again, no association could be observed between the number $CD8^+CD28^{null}$ T cells and the risk for EAR (HR: 0.99, p=0.415). Next to this, only the percentages of $CD4^+CD28^{null}$ T cells were associated with the risk for EAR (S2 Table). Higher percentages of these cells were also associated with a lower risk for EAR (HR: 0.91; p=0.036).

Furthermore, we generated a Kaplan-Meier curve with the EAR free survival stratified for the tertiles of $CD4^+CD28^{null}$ T cells (Figure 3). This analysis showed that high numbers of these cells were correlated with a significant higher EAR free survival rate compared with intermediate and low numbers of these cells (p=0.008 and p=0.009 respectively).

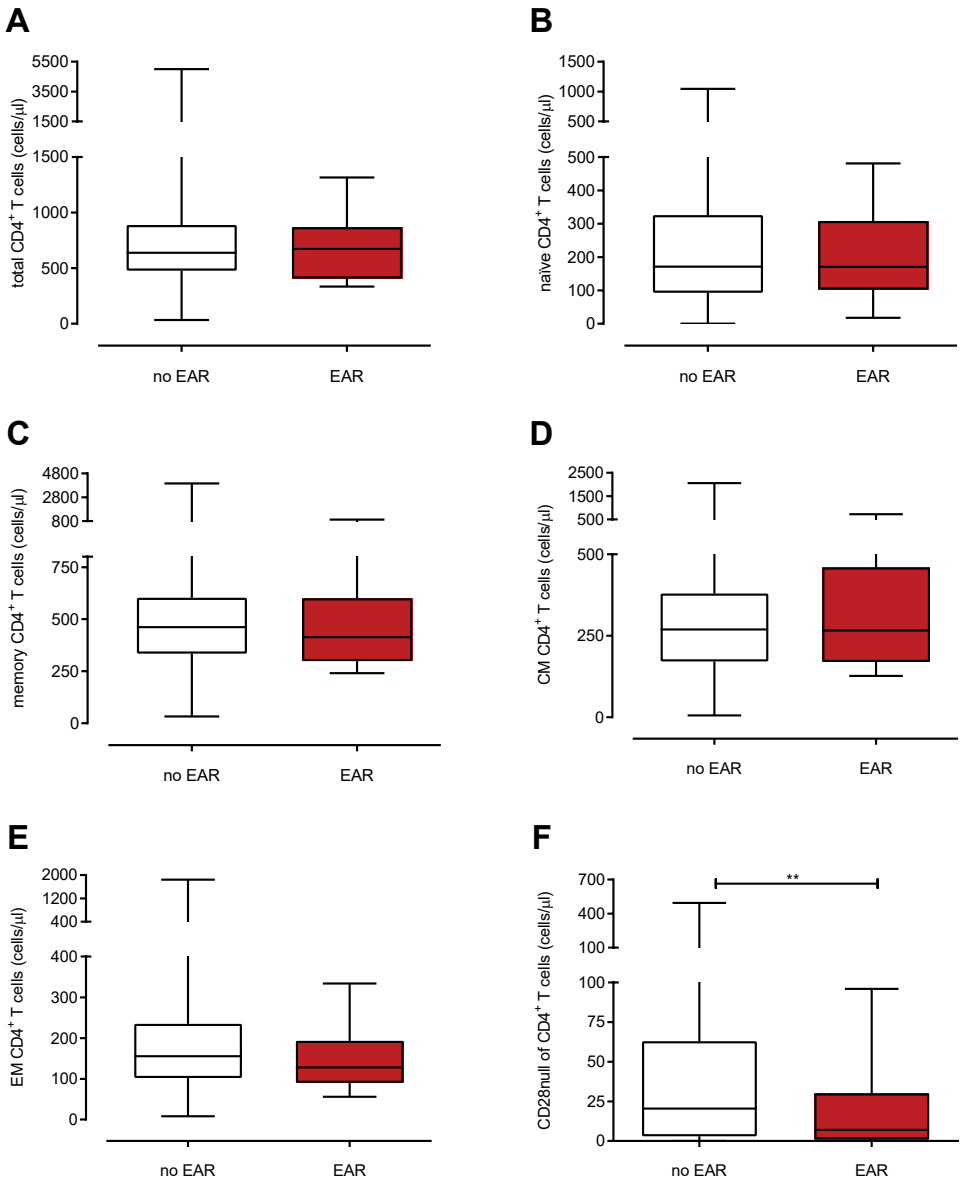


Figure 1. CD4⁺ T-cell differentiation status prior to KT. Absolute numbers of (A) total, (B) naive, (C) memory, (D) CM, (E) EM and (F) $CD28^{null}$ $CD4^{+}$ T cells are shown for the no rejection (white boxplot, *n*=192) and EAR (red boxplot, *n*=30) group of patients. Significant differences were calculated and shown (*p<0.05, **p<0.01, **p<0.001).

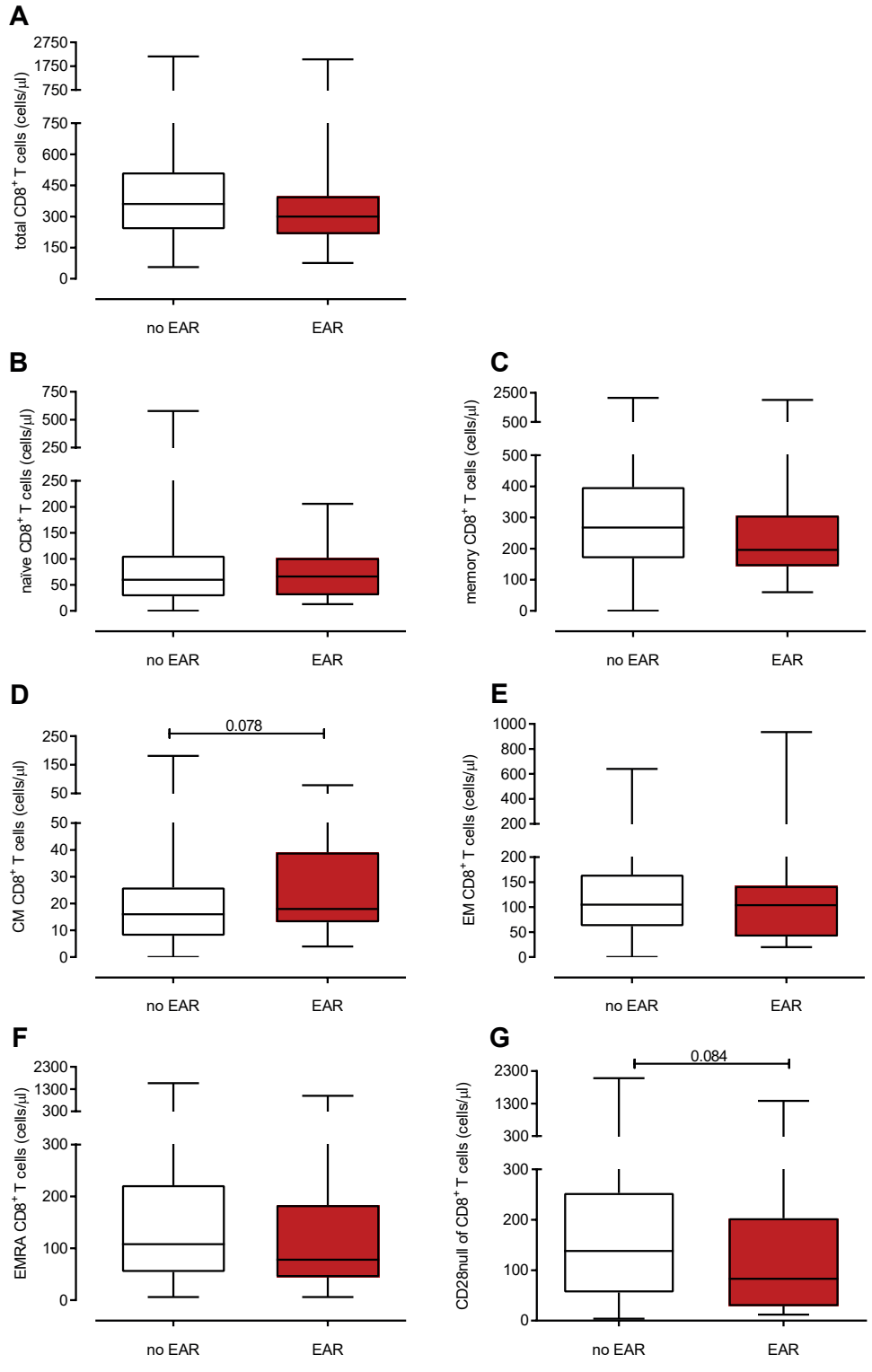


Figure 2. CD8⁺ T-cell differentiation status prior to KT. Absolute numbers of (A) total, (B) naive, (C) memory, (D) CM, (E) EM, (F) EMRA and (G) $CD28^{null}$ $CD8^{+}$ T cells are shown for the no rejection (white boxplot, *n*=192) and EAR (red boxplot, *n*=30) group of patients. Significant differences were calculated and shown (*p<0.05, **p<0.01, **p<0.001).

Table 2. RTL and RTEs content before kidney transplantation in patients with or without rejection within the first 3 months

KT Patients (n=222)	No Rejection (n=92) (86%)	Early Rejection (n=30) (14%)	P
RTL of CD4⁺ T cells	12.1 (9.1 – 15.0)	11.5 (10.0 – 13.3)	0.90
RTL of CD8⁺ T cells	11.4 (9.2 – 15.3)	11.1 (10.4 – 14.4)	0.83
CD31⁺CD4⁺ naive T-cell numbers (/µl)	106.5 (56.7 – 207.4)	104.3 (77.0 – 192.9)	0.99
CD31⁺ within CD4⁺ naive T-cells (%)	66.4 (55.1 – 75.1)	62.2 (53.7 – 76.8)	0.69
CD31⁺CD8⁺ naive T-cell numbers (/µl)	55.4 (24.1 – 566.3)	65.0 (12.8 – 203.9)	0.80
CD31⁺ within CD8⁺ naive T-cells (%)	97.7 (94.4 – 98.9)	97.9 (93.9 – 99.5)	0.41

Data are presented as medians (interquartile range). RTL: Relative Telomere Length, RTEs: Recent thymic emigrants.

Table 3. Hazard ratios for the clinical characteristics in relation to early acute allograft rejection (univariate analysis).

	HR	95%CI	P
Age donor (decades)	1.41	1.09 – 1.85	**0.011**
PRA historic (%)	1.11	1.04 – 1.18	**0.001**
Genetically related KT	0.36	0.15 – 0.88	**0.025**
CD4 positive CD28null T cells (cells/µL)	0.65	0.45 – 0.95	**0.025**
CD8 positive CD28null T cells (cells/µL)	0.98	0.94 – 1.03	0.420
CD4 positive central memory T cells (%)	1.02	1.00 – 1.04	0.124

*P<0.05, **P<0.01, ***P<0.001. CI: confidence interval, HR: hazard ratio. Age of the donor is presented in decades, PRA historic is presented with steps of 5%, and the CD4 positive and the CD8 positive CD28^{null} cells are presented with steps of 20 cells/µL.

Table 4. HRs for the T-cell parameters in relation to early acute allograft rejection (multivariate analysis).

	HR	95%CI	P
Age donor (decades)	1.40	1.06 – 1.84	**0.016**
PRA historic (%)	1.13	1.06 – 1.21	**<0.001**
Genetically related KT	0.47	0.19 – 1.17	0.102
CD4 positive CD28null T cells (cells/µL)	0.65	0.45 – 0.96	**0.028**

*P<0.05, **P<0.01, ***P<0.001. CI: confidence interval, HR: hazard ratio. The CD4 positive and the CD8 positive CD28^{null} cells are presented with steps of 20 cells/µL. Covariates: age donor, PRA historic, related KT.

Alloantigen-specific T cells predominantly co-express CD28

To obtain more insight between the association of highly differentiated T cells and the risk for EAR, a multiparameter flowcytometric assay (i.e. combining intracellular cytokine staining with cell surface markers) (72) was performed upon alloantigen stimulation

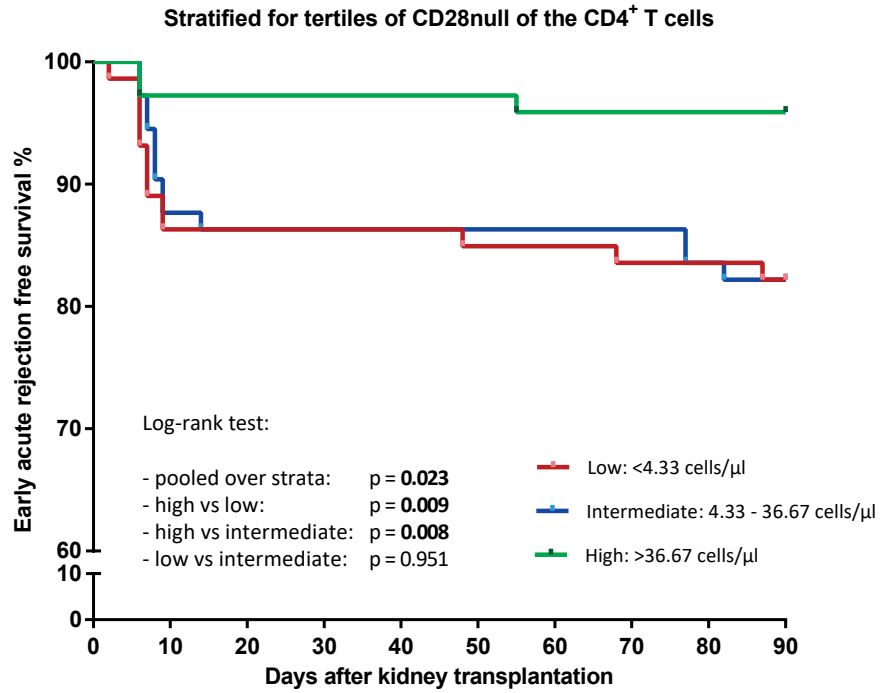


Figure 3. Tertiles of CD4⁺CD28^{null} T cells and EAR free survival. Kaplan-Meier curve representing EAR free survival rate against time after kidney transplantation in days. The curves represent the tertiles of CD4⁺CD28^{null} T cells. Low represents <4.33 cells/µl, intermediate represents 4.33–36.67 cells/µl and high represents >36.67 cells/µl. These groups were created based upon the frequencies of the cell numbers in our cohort and were divided into three equal groups.

of PBMCs obtained from kidney transplant recipients prior to kidney transplantation (Figure 4). In S2 Figure a representative example of the gating strategy is shown of the dissection of alloantigen-specific (CD137⁺ and CD137⁺ IL-2⁺ or IFN-γ⁺) CD4⁺ T cells into a CD28^{null} or CD28⁺ T-cell compartment. A similar FACS-analysis was performed for the CD8⁺ T-cell population. All CD3⁺ T cells are of patient's origin, as the donor cells were depleted of T cells prior to stimulation.

First, the frequency of alloantigen-specific CD137-expressing T cells was determined in the CD4⁺ T-cell compartment and these were mainly CD28⁺ (i.e. 72.4% vs 27.6%, p<0.001, Figure 4A). In addition, the CD28⁺ T cells contained more IL-2 (86.9% vs 13.1%, p<0.001) and IFN-γ (i.e. 65.1% vs. 34.9%, p<0.001) producing alloantigen-specific T cells compared to the CD28^{null} fraction (Figure 4B and 4C) upon alloantigen-stimulation.

Similar results were found for the CD8⁺ T cells. Upon alloantigen-stimulation, the CD137⁺CD8⁺ T-cells were mainly located within the CD28⁺ T cells (i.e. 60.4% vs 39.6%, p<0.001, Figure 4D). A higher proportion of these cells were also able to produce more IL-2 compared to their CD28^{null} counterparts (i.e. 72.4% vs 27.6%, p<0.001) and IFN-γ (i.e. 57.4% vs 42.6%, p<0.01) (Figure 4E and 4F).

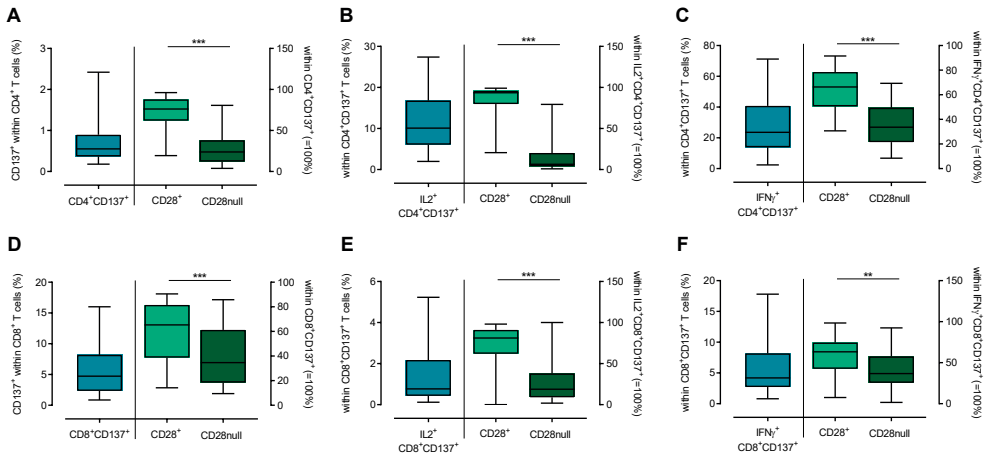


Figure 4. Cytokine producing alloantigen-stimulated T cells. First, the frequency of CD137+ cells within CD4+ T-cell population was determined (blue boxplot) in ESRD patients. These cells were divided into (A) a CD28+ (light green boxplot) and a CD28null subset (dark green boxplot). Next, the frequency of IL-2+ cells within the CD137+CD4+ was determined (blue boxplot). Furthermore, these cells were divided into (B) a CD28+ (light green boxplot) and a CD28null subset (dark green boxplot). Also the frequency of IFN-γ+ CD137+CD4+ T cells was determined (blue boxplot) and also these cells were divided into (C) a CD28+ (light green boxplot) and a CD28null subset (dark green boxplot). Next to the CD4+, the frequency of CD137+ cells within the CD8+ T-cell population was determined (blue boxplot) and divided into a CD28+ (light green boxplot) and a CD28null subset (dark green boxplot) (D). Within these CD137+CD8+ T cells, the frequency of IL-2 (F) and IFN-γ+ (G) was determined (blue boxplots) and divided into a CD28+ (light green boxplots) and a CD28null subset (dark green boxplots) (E+F). Significant differences were calculated and shown (*p<0.05, **p<0.01, **p<0.001).

DISCUSSION

In this study we analyzed within a large homogenous cohort of patients whether the degree of premature T-cell ageing prior to KT is associated with the risk for EAR post-KT. Of the three T-cell ageing parameters (thymic output, differentiation status and telomere length) used for the assessment of an immunological T-cell age, only the differentiation status was associated with the risk for EAR. A higher number as well as percentage of CD28null T cells, mainly within the CD4+ T-cell population, is associated with a lower risk for EAR. The number of RTEs or the relative telomere length of CD4+ and CD8+ T cells were both not associated with the risk for rejection.

CD28null T cells are predominantly located within the (antigen-experienced) memory population and in particular within the more differentiated T cells (73). Loss of CD28 on the cell surface of (CD4+) T cells is one of the features of T-cell ageing as CD28null T cells are present at a low frequency and rarely found in young individuals. Moreover, CD4+CD28null T cells are highly associated with seropositivity for cytomegalovirus (CMV) (74-76). However, in this study we did not observe a significant difference with respect to frequencies of CMV-seropositive individuals between the EAR group and the no rejection group.

The majority of CD28null T cells lack the expression of CCR7. This is important for homing to secondary lymphoid organs (31) where (naive) T cells are activated by antigen-presenting cells presenting alloantigens in a direct or indirect manner. In addition to the lack of CD28, they are less able to provide co-stimulation through the CD40L-CD40 pathway contributing to defective helper function (77).

A characteristic feature of CD4+CD28null T cells is their restricted T-cell receptor profile (34, 78) compared to the CD28+ T-cell population. This may compromise their reactivity to foreign antigens like for example alloantigens. Thus, theoretically higher numbers of CD28null T cells might result in lower alloreactivity. This hypothesis is supported by our finding that IL-2 and IFN-γ producing alloantigen-specific T cells were predominately located within the CD28+ T-cell population, both within the CD4+ and the CD8+ T-cell compartment. In addition, in liver transplant recipients, higher frequencies of CD4+CD28+ T cells were found in allograft rejecting patients (79). Next to this, the higher percentages of CD4+ and CD8+ CM T cells in the EAR group, supports this finding by suggesting that the less differentiated cells are more present in patients who develop an acute rejection.

The finding that high CD28null T-cell numbers are associated with a lower risk for allograft rejection is in line with an earlier small study in which these cells were shown to have an exhausted phenotype (67). Furthermore, several studies support the senescent character of these CD28null T cells. A study by Nunes et al. showed that highly differentiated CD8+ T cells were accompanied by an increase of CD28null and CD27− T cells (80). In line with this, the highly differentiated CD8+ T cells were also associated with PD-1 positivity, which was in accordance with replicative senescence of these T cells (80). Next to this, a study by Dirks et al. showed that CMV viremia was associated with increased PD-1 expression on CD4+CD28nullCD27− T cells in transplant recipients (81). Furthermore, a study by Koch et al. showed that the expression of the senescence markers CD57 and KLRG-1 were more present in the highly differentiated CD4+ and CD8+ T cells, and were almost absent in the naive T-cell populations (26). Our group also showed that the percentage of CD57+ cells was significantly higher in CD4+ memory T cells (11). In a recent study, a lower frequency of CD4+CD28null T cells was also observed in relation to acute rejection within the first year after kidney transplantation but statistical significance was lost in the multivariate analysis (66). The patient population in that study was much more heterogeneous, including post-mortal kidney transplantations, more re-transplantations and a higher number of CMV-seropositive patients which could at least in part explain the difference in findings between both studies (66). The higher number of CMV-seropositive patients in that study might also explain why we could not find relations between CD8+ EMRA T-cells and the development of EAR in our current study.

Compared to the CD28+ T-cell population, the CD28null T cells are known to have shorter telomeres (74). The fact that we could not detect a correlation between the overall

telomere length and the risk for EAR, might be explained by the relatively low frequency of $CD28^{null}$ T cells within the $CD4^+$ T-cell population in which the relative telomere length was assessed. In line with our findings, a study by Oetting et al. could also not find an association between the RTL and the risk for acute rejection (82).

In this study we could not find an association between RTEs and the risk for EAR based on the expression of CD31. Since the thymus involutes rapidly after puberty, the contribution of the thymus to maintain the (naive) T-cell pool is relatively small in older individuals (83). Maintaining adequate numbers of naive T cells upon ageing mainly relies on homeostatic proliferation either through homeostatic cytokines like IL-7 or low-affinity T-cell receptor interactions with self-antigens being presented by antigen-presenting cells (84). Since the total number of naive T cells is not different between the two groups of patients, it is likely that the degree of homeostatic proliferation is similar. These findings suggest that the naive T-cell compartment is not of significant importance for alloreactivity within the first three months after KT and that the memory T-cell compartment is more relevant (85).

Another interesting finding in this study was the higher risk for EAR in patients with a higher historical PRA score. Several other studies have shown that high levels of historical PRA scores are associated with rejection and graft failure (86-88). Our findings support the results of these studies with regard to rejection and suggest that a peak historical PRA score might also be an important contributor in the setting of EAR in KT.

As the T-cell ageing parameters do not change post-KT (69), it is likely that at the time of rejection the composition of T cells including the frequency of $CD28^{null}$ T cells is similar to the pre-KT value. This means that allograft rejection risk assessment based on T-cell ageing prior to KT probably resembles the T-cell age prior to time of rejection.

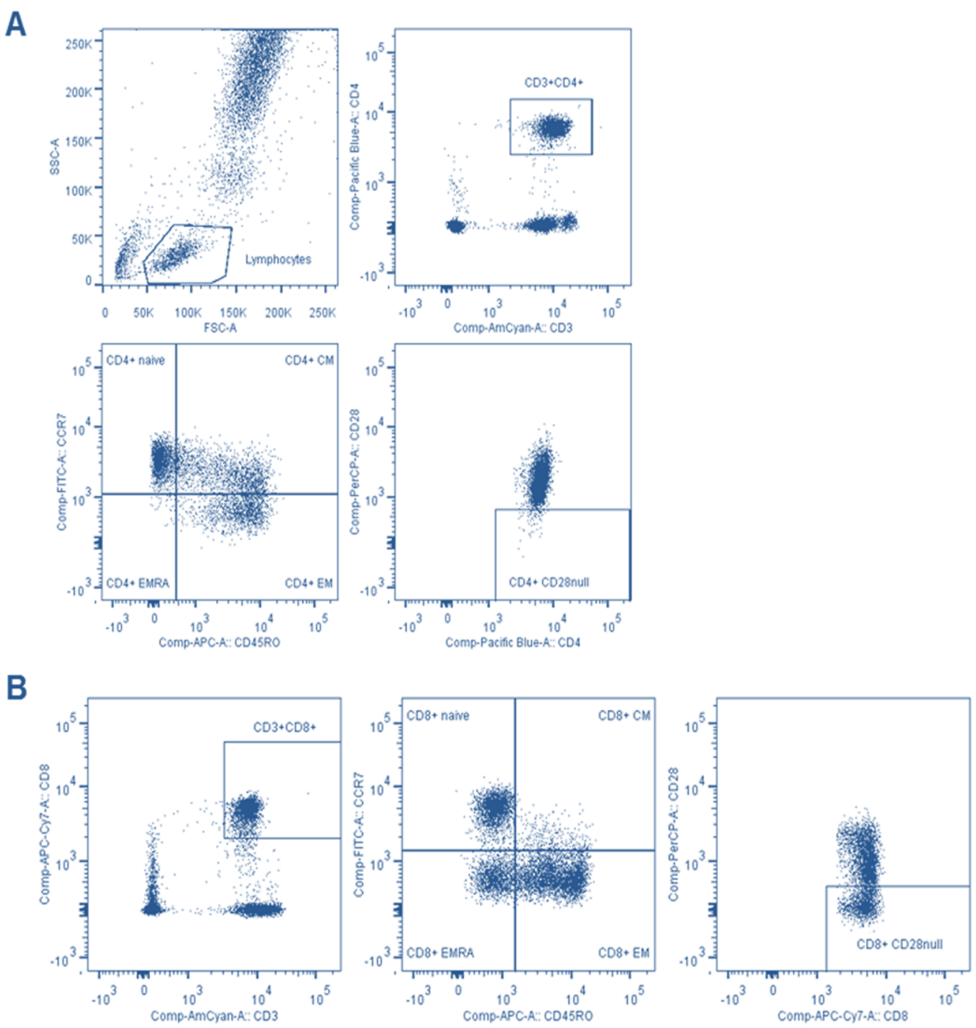
In conclusion, the T-cell ageing-related expansion of highly differentiated $CD4^+CD28^{null}$ T cells in ESRD patients is associated with a lower risk for EAR. This may be related to a significantly lower percentage of alloreactive T cells within the $CD28^{null}$ T cell fraction. This study provides in depth analysis of the various T-cell subsets with regard to T-cell ageing in the setting of KT. To give a more accurate representation we mainly focused on absolute cell numbers instead of percentages. Furthermore proliferative history was also taken into account by the assessment of RTL. These characteristics were then combined with functional capacities of the highly differentiated $CD28^{null}$ T cells and associations were drawn with regard to EAR. The patient characteristics of the two patient groups are highly similar, which makes the study population homogenous. Next to this, only living-donor KT was considered. We believe that the combination of the aforementioned aspects contribute novel facets in the relationship between premature T-cell ageing and the risk for EAR after KT. Further analyses are needed to investigate the properties of these cells and to define cut-off values for the clinical practice. In the future this could possibly attribute to an optimal risk assessment for a personalized immunosuppressive regimen.

DISCLOSURES

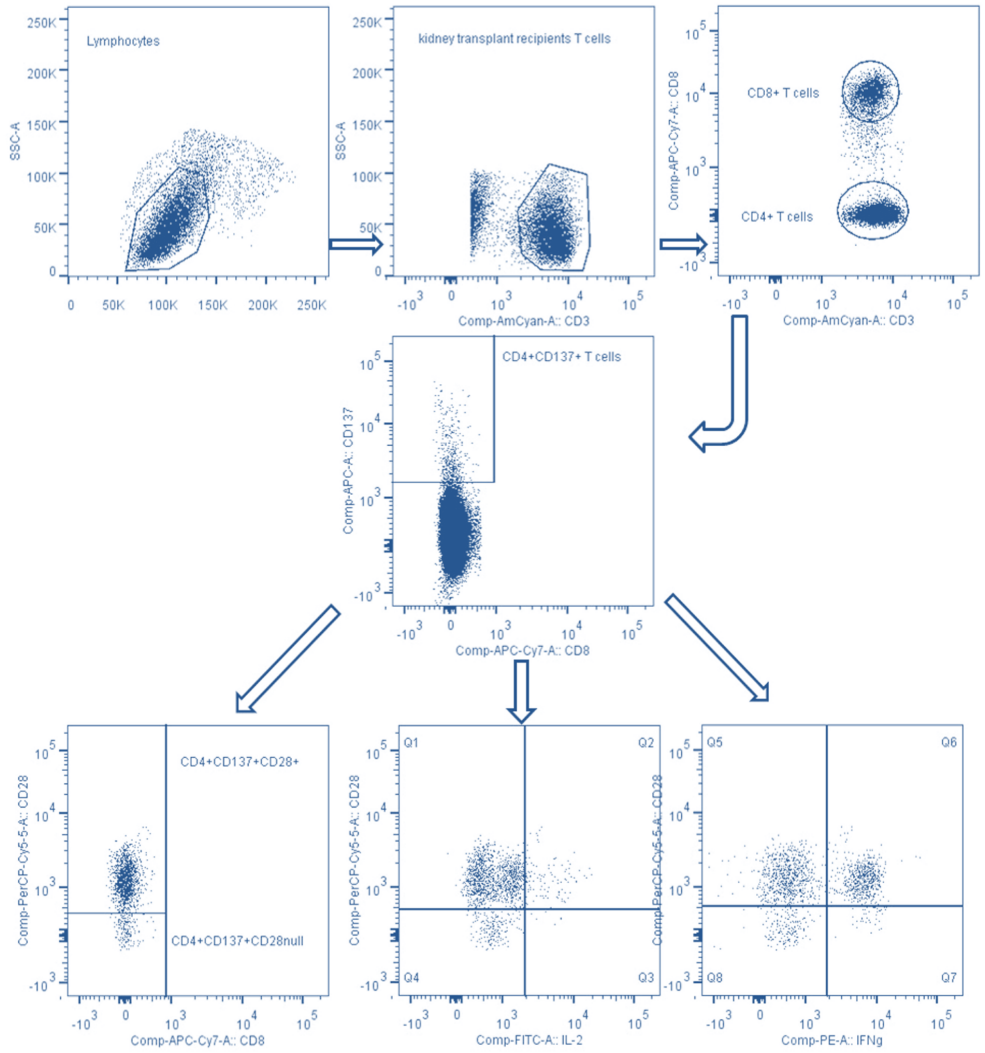
The authors declare no competing financial and commercial interests. This study was funded by the Dutch Kidney Foundation (KSPB.10.12).

2
LOSS OF CD28 ON PERIPHERAL T CELLS DECREASES THE RISK FOR EARLY ACUTE REJECTION AFTER KIDNEY TRANSPLANTATION

SUPPLEMENTARY FILES



Supplementary Figure 1. Gating strategy of (A) CD4⁺ and (B) CD8⁺ T cells. A typical example of the gating strategy is shown. Briefly, lymphocytes were identified based on the forward/sideward characteristics followed by the selection of the $CD3^+CD4^+$ T cells. These $CD4^+$ T cells were dissected into subsets using CCR7 and CD45RO. Furthermore, the number of $CD28^{null}$ cells was examined within the (A) $CD4^+$ T-cell population. The same strategy was followed for (B) $CD8^+$ T cells.



Supplementary Figure 2. Example of the gating strategy of cytokine producing alloantigen-stimulated T cells. Briefly, lymphocytes were identified based on the forward/sideward characteristics. Next the $CD3^+CD4^+$ (i.e. $CD8^-$) and $CD3^+CD8^+$ were selected and within these, the $CD137^+$ were selected as shown for the $CD4^+$ population. These cells were divided into a $CD28^+$ and $CD28^{null}$ population. Furthermore the frequency of IL-2^+ and IFN-γ^+ $CD137^+CD4^+$ was determined and also dissected into a $CD28^+$ and $CD28^{null}$ subset. A similar approach was applied for the $CD8^+$ T-cell compartment.

Supplementary Table 1. T-cell differentiation status before kidney transplantation in patients with or without rejection within the first 3 months

KT Patients (n=222)	No Rejection (n=192) (86%)	Early Rejection (n=30) (14%)	P
$CD4^+$ naive T cells (%)	29.4 (18.0 – 42.0)	29.7 (24.1 – 36.4)	0.905
$CD4^+$ memory T cells (%)	70.6 (60.0 – 82.0)	70.3 (63.6 – 75.9)	0.905
$CD4^+$ central memory T cells (%)	40.5 (31.3 – 50.6)	46.6 (37.3 – 54.6)	0.055
$CD4^+$ effector memory T cells (%)	24.4 (16.5 – 32.5)	24.9 (14.6 – 27.7)	0.303
$CD4^+CD28^{null}$ T cells (%)	3.0 (0.5 – 9.6)	1.0 (0.2 – 4.1)	**0.011**
$CD8^+$ naive T cells (%)	18.1 (8.8 – 35.1)	22.8 (13.8 – 39.9)	0.323
$CD8^+$ memory T cells (%)	81.9 (65.0 – 91.2)	77.3 (60.1 – 86.2)	0.323
$CD8^+$ central memory T cells (%)	4.2 (2.3 – 7.9)	6.1 (4.1 - 13.6)	**0.005**
$CD8^+$ effector memory T cells (%)	32.7 (20.3 – 45.4)	28.2 (23.8 - 41.6)	0.899
$CD8^+$ EMRA T cells (%)	33.7 (18.5 - 52.1)	31.6 (16.9 - 50.3)	0.759
$CD8^+CD28^{null}$ T cells (%)	40.5 (19.6 - 60.4)	27.6 (19.6 - 52.9)	0.190

Data are presented as medians (interquartile range).

Supplementary Table 2. Hazard ratios for the clinical characteristics in relation to early acute allograft rejection (multivariate analysis)

	HR	95%CI	P
Age donor (decades)	1.43	1.09 – 1.88	**0.011**
PRA historic (%)	1.13	1.07 – 1.22	**<0.001**
Genetically related KT	0.51	0.21 – 1.28	0.152
CD4 positive $CD28^{null}$ T cells (%)	0.91	0.84 – 0.99	**0.036**

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CI: confidence interval, HR: hazard ratio. Age of the donor is presented in decades, PRA historic is presented with steps of 5%, the CD4 positive and the CD8 positive CD28null cells are presented with steps of 20 cells/μL, CD4 and CD8 positive central memory T cells are presented with steps of 1%, and the CD4 positive CD28 null T cells are presented with steps of 1%

Chapter 3

$CD4^+$ $CD28^{NULL}$ T CELLS ARE NOT ALLOREACTIVE UNLESS STIMULATED BY IL-15

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ABSTRACT

Background

Proinflammatory, cytotoxic CD4+CD28null T cells can be substantially expanded in patients with end-stage renal disease. These cells have been associated with the risk for rejection, but their alloreactive potential is unknown.

Methods

CD4+CD28null T cells were stimulated with HLA-mismatched antigen presenting cells in the absence/presence of exogenous cytokines. Alloreactive potential was evaluated by proliferation, degranulation, cytotoxicity and cytokine production. Furthermore, their suppressive capacity was assessed by measuring inhibition of proliferating alloreactive CD28+ T cells.

Results

CD4+CD28null T cells contained alloreactive (CD137+) T cells but did not proliferate in response to allogeneic stimulation, unless IL-15 was added. However, they could proliferate upon stimulation with CMV-antigen without exogenous cytokines. IL-15 increased the frequency of proliferating alloreactive CD4+CD28null T cells to 30.5% without inducing CD28 expression ($p<0.05$). After allogeneic stimulation together with IL-15 and IL-21, frequency of degranulating CD107a+CD4+CD28null T cells increased significantly from 0.6% to 5.8% ($p<0.001$). Granzyme B and perforin positivity remained similar, but production of IFN-γ and TNF-α increased by the combination of IL-15 and IL-21 ($p<0.001$ and $p<0.05$, respectively). Finally, CD4+CD28null T cells did not show significant suppression.

Conclusion

CD4+CD28null T cells represent a population with absent alloreactivity unless IL-15 is present.

INTRODUCTION

Patients with end-stage renal disease (ESRD) have a prematurely aged circulatory T-cell system (11, 13). The discrepancy between their chronological and their immunological T-cell age can be as much as 15-20 years (11, 13). A typical feature of an aged peripheral T-cell system is the increase in late differentiated memory T cells (20, 21, 89). A key characteristic of these cells is loss of the co-stimulatory molecule CD28 on their surface (20, 23, 27, 29). These CD28null T cells are mainly of effector memory (CCR7-CD45RO+) and EMRA phenotype (CCR7-CD45RO- but CD45RA+) (30, 90). CD4+ and CD8+ T cells can both lack CD28 (23, 27, 29, 30). In patients with ESRD, there is a significant expansion of the CD28null T-cell populations and the frequency of CD4+CD28null T cells may be as high as 65% (30).

Continuous antigenic stimulation and/or a proinflammatory environment are probably the main drivers of CD28 loss (91). Increased CD28null T-cell frequencies are therefore found in patients with ESRD, HIV infection, rheumatoid arthritis and type 1 diabetes (27, 29, 30). Additionally, CMV infection causes a persistent expansion of terminally differentiated CD28null T cells (42, 43).

CD4+CD28null T cells are actually not classical helper T cells but share many functional similarities with cytotoxic CD8+ T cells. CD4+CD28null T cells produce proinflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and contain cytotoxic molecules like granzyme B and perforin in intracellular stores (23, 29-32).

Given the highly proinflammatory and cytotoxic profile of CD4+CD28null T cells, any alloreactivity of these cells may be particularly harmful for the transplanted organ. Indeed, the presence of these cells has been associated with the risk for development of (co-stimulatory blockade-resistant) rejection after renal transplantation (RT) (29, 34, 36, 92). Association between increased numbers of these cells prior to RT and a reduced risk for early acute allograft rejection was also a part of these observations (36). However, in vitro studies about the alloreactivity of CD4+CD28null T cells are lacking, but are needed for a proper understanding of the role of these cells with regard to rejection. Therefore, in this study the alloreactive potential and immunomodulatory properties of CD4+CD28null T cells were examined.

MATERIALS AND METHODS

Study population

Samples were obtained from renal transplant recipients who received a renal allograft in the period from January 2011 to August 2013. All patients gave written informed consent to participate in this study. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2012-022) and was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Clinical variables were determined as shown in Table 1.

Responder and stimulator PBMC isolation

Blood was obtained from renal transplant recipients one day prior to RT and from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by using Ficoll-Paque Plus (GE healthcare, Uppsala, Sweden). Isolated PBMCs of responders were stored at –150°C with ≥10×10^6 cells/vial whereas stimulator PBMCs were further processed as described below.

Preparation of stimulator

Selection of healthy donors was based on ≥2 HLA-I mismatches and ≥1 HLA-II mismatches with the responder. PBMCs of these donors were labeled with CD3-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently depleted for T cells using the autoMACS® Pro (Miltenyi Biotec). Allogeneic stimuli were depleted for >95% of CD3$^+$ T cells as assessed by flow cytometry upon staining the negative fraction with Amcyan-labeled anti-CD3 (BD, Erembodegem, Belgium). CD3-depleted cells (stimulator) were stored at –150°C at 5×10^6 cells/vial until further use.

Cell sorting of responder cells

PBMCs of 11 renal transplant recipients, with a frequency of ≥10% CD28^{null} T cells within CD4$^+$ T cells, were selected. These PBMCs were thawed and prepared for sorting experiments using the following antibodies: phycoerythrin-cyanine 7 (PE-Cy7)-labeled anti-CD3 (BD Pharmingen), brilliant violet 510 (BV510)-labeled anti-CD4 (Biolegend, San Diego, CA, USA), phycoerythrin (PE)-labeled anti CD28 (BD) and a live-dead marker 7-amino-actinomycin D (7-AAD or ViaProbe, BD). After the staining procedure, cells were washed and resuspended at 20–25×10^6/ml and sorted on the FACSAria II SORP (BD) into CD4$^+$CD28^{null} and CD4$^+$CD28$^+$ T cells. Isolated T-cell fractions were used as responder cells and subjected to allogeneic stimulation. Median purities were 97.0% [94.9%–98.8%] for CD4$^+$CD28^{null} T cells.

Stimulation

In each experiment two different allogeneic stimuli (≥2 HLA-I mismatches and ≥1 HLA-II mismatches) were included, and used in a one way mixed lymphocyte reaction (MLR).

PBMC and sorted CD4$^+$CD28$^+$ and CD4$^+$CD28^{null} T cells (responders) were labeled with CFSE (Molecular Probes®, Leiden, the Netherlands) according to manufacturer's instruction and stimulated in triplicate at 5×10^4/well with an allogeneic stimulus (40Gy-irradiated) at a 1:1 ratio for 6 days. In a set of experiments, PBMCs of CMV-seropositive individuals were either not or pre-incubated with humanized anti-IL-15R antibody (5µg/ml; Thermo Fisher, Bleiswijk, the Netherlands) and/or humanized anti-IL-21R antibody (10µg/ml; Pfizer, New York, USA) and subsequently stimulated with CMV-lysate (30 µg/ml; Microbix Biosystems Inc. Ontario, Canada). These concentrations are known to provide sufficient blockade of the receptors according to the certificate of analysis provided by Thermo Fisher (IL-15R blockade) and a study by de Leur et al. (93) (IL-21R blockade).

Allogeneic stimulation was performed with/without IL-15 (10 ng/ml, Peprotech Inc., London, UK) and/or IL-21 (10 ng/ml, Peprotech Inc.). These cytokines were based upon our experiments (see results) and studies showing the effect of IL-15 and IL-21 on memory T cells (94-96). Ten ng/ml was chosen, because no differences were seen between stimulation with 10 or 50 ng/ml (data not shown). As a negative control, responders were stimulated using only culture medium (RPMI-1640 (Lonza Group, Basel, Switzerland) with 10% heat-inactivated pooled human serum). On day 5, wells of the same condition were pooled, supernatant stored at -80°C and 5 µg/mL monensin (Golgistop; BD Pharmingen) was added overnight. On day 6, alloreactive potential including proliferation, degranulation, cytotoxicity and cytokine production was analyzed as described in the sections below.

Allogeneic proliferation

Proliferation was analyzed by measuring CFSE dilution and determining the frequency of CFSE$^-$ T cells. Cells were stained using the following antibodies; PE-Cy7-labeled anti-CD3 (BD Pharmingen), BV510-labeled anti-CD4 (Biolegend) and PE-labeled anti-CD28 (BD). A dump-channel was employed, in order to exclude unwanted cells by co-staining the cells for the live-dead marker 7-AAD, peridin chlorophyll protein (PerCP)-labeled anti-CD19 (BD), PerCP-Cy5.5-labeled anti-CD56 (Biolegend) and PerCP-labeled anti-CD14 (BD).

Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD).

CD137 expression

Alloantigen-specific T cells were determined by the CD137 multi-parameter flowcytometric assay as published previously (71). Briefly, PBMCs of 10 patients were stimulated with T-cell depleted donor or autologous PBMCs (control) at a 1:1 ratio in presence of αCD49d (as co-stimulation) at 1 µg/mL (BD) for 24 hours. During the last 12 hours, golgiplug (Brefeldin A; BD) and golgistop (Monensin; BD) were added. Cell surface was stained with the following antibodies; AmCyan-labeled anti-CD3 (BD), allophycocyanin (APC)-Cy7-labeled anti-CD8 (BD) and PerCP-Cy5.5-labeled anti-CD28 (BD). After fixation and permeabilization, CD137 was stained intracellularly by using APC-labeled anti-CD137 (BD). Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD). Allogeneic CD137-expressing CD4$^+$CD28$^+$ or CD4$^+$CD28^{null} T cells were corrected for CD137-expressing cells upon autologous stimulation.

Degranulation and cytotoxic potential upon allogeneic stimulation

Following 6 days of stimulation, cells were harvested and stained as described above, and combined with allophycocyanin (APC)-labeled anti-CD107a (BD Pharmingen). Degranulation was determined by analyzing the percentage of CD107a$^+$CD4$^+$CD28^{null} T cells. In addition, a dissection was made in order to analyze CD107a within proliferating (CFSE$^-$) as well as non-proliferating (CFSE$^+$) CD4$^+$ T cells. Samples were measured on the FACSCanto II (BD) analyzed using FACS Diva software version 6.1.2 (BD).

Cytotoxic potential was analyzed by measuring granzyme B+ and perforin+ CD4+CD28null T cells. Briefly, a one way MLR was performed with sorted CFSE-labeled CD4+CD28null T cells, stimulated with different HLA-mismatched irradiated (40Gy) CD3-depleted cells alone or supplemented with exogenous IL-15 and IL-21. As a negative control, a stimulation using only culture medium was included. Upon cell surface staining as described earlier, cells were fixed and permeabilized. Subsequently, granzyme B and perforin were stained intracellularly using Alexa Fluor 647-labeled anti-granzyme B (Biolegend) and BV421-labeled anti-perforin (Biolegend). Samples were measured on the FACSCanto II (BD) and analyzed with FACS Diva software version 6.1.2 (BD).

Analysis of cytokine production

The concentration of cytokines, i.e. IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A, present within collected supernatants, was determined using the human Th1/Th2/Th17 cytometric bead array (CBA) cytokine kit (BD) according to manufacturer's instruction. Briefly, cytokine standards with fixed concentrations of each analyte were measured on the FACS Canto II (BD) and median fluorescence intensities (MFI) were used to generate a standard curve for each analyte, using a 4-parameter logistic regression analysis with GraphPad Prism 5 (CA, USA). MFI of the various analytes within the samples were transformed into concentrations (pg/mL) of a particular cytokine. For these experiments, we focused on production of proinflammatory cytokines TNF-α and IFN-γ.

Analysis of suppression

Patient PBMCs were sorted into CD4+CD28null T cells and T cells without CD4+CD28null (responders). CD4+CD28null T cells were evaluated for suppressive potential of allogeneic-induced proliferation of the responder fraction consisting of CD8+ and CD4+CD28+ T cells. Suppressive capacity of CD4+CD28null T cells was either tested directly or upon allogeneic expansion in presence of both IL-15 (50 ng/mL) and IL-21 (50 ng/ml). Briefly, responders were CFSE-labeled, and either not or stimulated in triplicate at $5x10^4$/well with irradiated allogeneic stimuli at a 1:1 ratio, in absence or presence of CD4+CD28null T cells (expanded using the same allogeneic stimulus). A 1:2 suppressor:responder ratio was used when CD4+CD28null T cells were not expanded. After 6 days, suppressive capacity was analyzed by evaluating percentages of CFSE− responders in presence or absence of CD4+CD28null T cells.

Statistical analysis

All variables are presented as medians (with interquartile ranges). Differences between two paired conditions were analyzed using the Wilcoxon signed rank test. Differences between two different fractions (e.g. divided vs undivided fractions) were analyzed using the Mann-Whitney U test. Differences between three or more paired conditions were analyzed using the Friedman test followed by a correction for multiple testing.

The significance level (p-value) was two-tailed and an α of 0.05 was used for all analyses. Statistical analyses were performed using SPSS® version 21.0 for Windows® (SPSS Inc., IL, USA) and GraphPad Prism 5 (CA, USA). Figures were created with GraphPad Prism 5 (CA, USA).

RESULTS

Study population

In total 11 patients were included (Table 1). Median patient age was 60 years. Seven patients were male and all patients were CMV IgG+. Cause of renal failure was nephrosclerosis/atherosclerosis/hypertension (3 patients), focal segmental glomerulosclerosis (2 patients), diabetes mellitus type 2 (2 patients), vasculitis, polycystic kidney disease, hemolytic-uremic syndrome and one unknown. Six patients underwent dialysis prior to transplantation. Median frequency of CD4+CD28null T cells prior to RT was 31.3% (17.8%-36.4%).

CD4+CD28null T cells need exogenous cytokines to proliferate upon allogeneic stimulation

CD4+CD28null T cells showed no increase in proliferation upon allogeneic stimulation compared with CD4+CD28+ T cells (Figure 1A-B). Median percentage of proliferating CD4+CD28null T cells remained around 1.7% (Figure 1B), while this percentage rose significantly from 1.9% to 25.7% ($p<0.001$) for CD4+CD28+ T cells (Figure 1B). An obvious explanation for this difference could be that the sorting procedure had a deleterious effect on the proliferative capacity of CD4+CD28null T cells. Therefore, first we assessed CD28 expression on proliferating alloreactive CD28+ T cells. CD28 expression appeared to be slightly higher after allogeneic stimulation ($p<0.05$), without significant induction of CD28null T cells (Figure 1C). Then proliferation of CD28null T cells in the unsorted T-cell fraction was analyzed, which confirmed that CD28null T cells indeed do not proliferate in presence of allogeneic stimulator cells (Figure 1D). The CD28null T-cell population also harbors CMV-specific T cells. In presence of CMV-antigen, CD4+CD28null T cells showed significant proliferation (Figure 1E). Median percentage of proliferating CD4+CD28null T cells rose from 7.7% to 70.0% ($p<0.05$; $n=8$) upon stimulation with CMV-antigen. Blocking the IL-15R and/or IL-21R did not influence CMV-lysate-induced proliferation of CD4+CD28null T cells, indicating that it is independent from IL-15 or IL-21 (data not shown). Thus, CD4+CD28null T cells are not senescent and are able to proliferate vigorously upon recognition of a cognate antigen.

CD137 expression is an early and sensitive marker of antigen-specific T-cell activation and can be used to identify the total pool of alloreactive T cells (71). Using this assay, presence of alloreactive CD137+ T cells was shown in both CD28null and CD28+ T cells (Figure 2A-B). Median percentage of cells within the CD28+ fraction was 0.36% and within the CD28null fraction 0.21% ($n=10$) (Figure 2B). As alloreactive T cells reside within CD4+CD28null T cells, we set out to evaluate whether exogenous cytokines could induce

Table 1. Patient characteristics

	RT Patients (n=11)
Age	60 (44 – 66)
Male	7 (64%)
CMV-seropositivity	11 (100%)
Underlying disease	
Nephrosclerosis/atherosclerosis/hypertension	3 (27%)
Focal segmental glomerulosclerosis	2 (18%)
Diabetes	2 (18%)
Vasculitis	1 (9%)
Polycystic Kidney Disease	1 (9%)
Hemolytic-uremic syndrome	1 (9%)
Unknown	1 (9%)
Dialysis prior to transplantation	6 (55%)

Data are presented as median (interquartile range) or as number (percentage).

proliferation of alloreactive CD4+CD28^{null} T cells. This showed that IL-15 and IL-21, which are cytokines known to stimulate memory T cells (94-96), provided the highest fold of increase of CD4+CD28^{null} T-cell frequency (Figure 2C). Based upon these results we continued to analyze the effect of the cytokines IL-15 with/without IL-21 at 10 ng/ml.

In presence of IL-15 alone ($p<0.05$), and both IL-15 and IL-21 ($p<0.001$), percentage of proliferating CD4+CD28^{null} T cells increased significantly upon allogeneic stimulation (Figure 2D). IL-15 increased the median percentage of proliferating CD4+CD28^{null} T cells to 30.5%, while the combination of both cytokines increased this to 48.6%. Thus, CD4+CD28^{null} T cells need primarily IL-15 to proliferate upon allogeneic stimulation. Increase in proliferation was due to a combination of allogeneic stimulation and the addition of exogenous cytokines. Stimulation with only IL-15 and IL-21 showed a significant lower proliferation than when an allogeneic stimulus was added. Median percentage of proliferating CD4+CD28^{null} T cells with only IL-15 amounted to 9.1% while this was significantly higher when an allogeneic stimulus was added

(30.5%, $p<0.05$; $n=6$). A similar result was found when IL-15 and IL-21 were combined. Median percentage of proliferating cells with only IL-15 and IL-21 amounted to 10.2%, while this was significantly higher when an allogeneic stimulus was present (30.0%, $p<0.05$; $n=6$).

Degranulation and upregulation of cytotoxic molecules by cytokines is limited

Degranulation was assessed by measuring CD107a expression. Median frequencies of CD107a+ T cells increased from 0.6% to 1.4% ($p<0.05$) after allogeneic stimulation. Adding only IL-15 or IL-21 to the culture did not have an effect on CD107a expression within CD4+CD28^{null} T cells (Figure 3A), unless both cytokines were combined. This increased the median CD107a expression to 5.8% (2.7%–8.5%, $p<0.001$) (Figure 3A). Subsequently, we dissected CD107a-expressing cells into those proliferating or not in response to allogeneic stimulation (Figure 3B). In absence of exogenous cytokines,

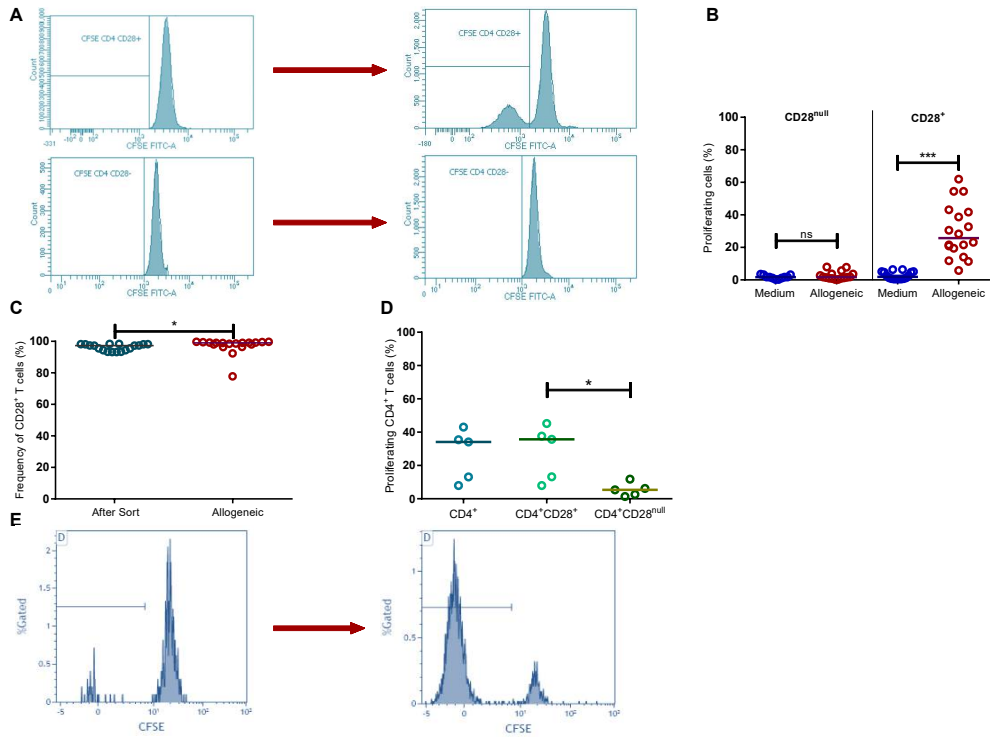

Figure 1. Proliferative capacity of CD4+CD28^{null} T cells. (A) An example of the absence of proliferation by CD4+CD28^{null} T cells (upper histograms) and the presence of proliferation by CD4+CD28+ T cells (lower histograms) from the same patient upon allogeneic stimulation is shown. The histograms on the left represent the unstimulated condition, while the histograms on the right represent the allogeneic condition. (B) The frequency of proliferating cells within sorted CD4+CD28^{null} T cells and sorted CD4+CD28+ T cells are shown after allogeneic stimulation. Nine patient samples were stimulated with 2 different allogeneic stimulators, but due to lack of material, only 12 tests could be analyzed pairwise for the CD4+CD28^{null} T cells and only 16 tests for CD4+CD28+ T cells. Differences between the conditions were analyzed with a Wilcoxon signed rank test. (C) The frequencies of CD4+CD28+ T cells are represented immediately after the sorting procedure and after allogeneic stimulation. Nine patients were stimulated with 2 different allogeneic stimulators separately. This led to $n=18$ for the CD4+CD28+ T cells for a Wilcoxon signed rank test. (D) Frequencies of proliferating CD4+ T cells within PBMCs from 5 patients after allogeneic stimulation are shown. (E) An example of proliferation by CD4+CD28^{null} T cells upon stimulation with CMV-antigen is shown. The histogram on the left represents the unstimulated condition, while the histogram on the left shows the CMV-antigen stimulated condition. Frequencies of cells are depicted as individual percentages as well as medians. Significant differences were calculated and shown (ns: not significant, *$p<0.05$, **$p\leq0.01$, ***$p\leq0.001$).

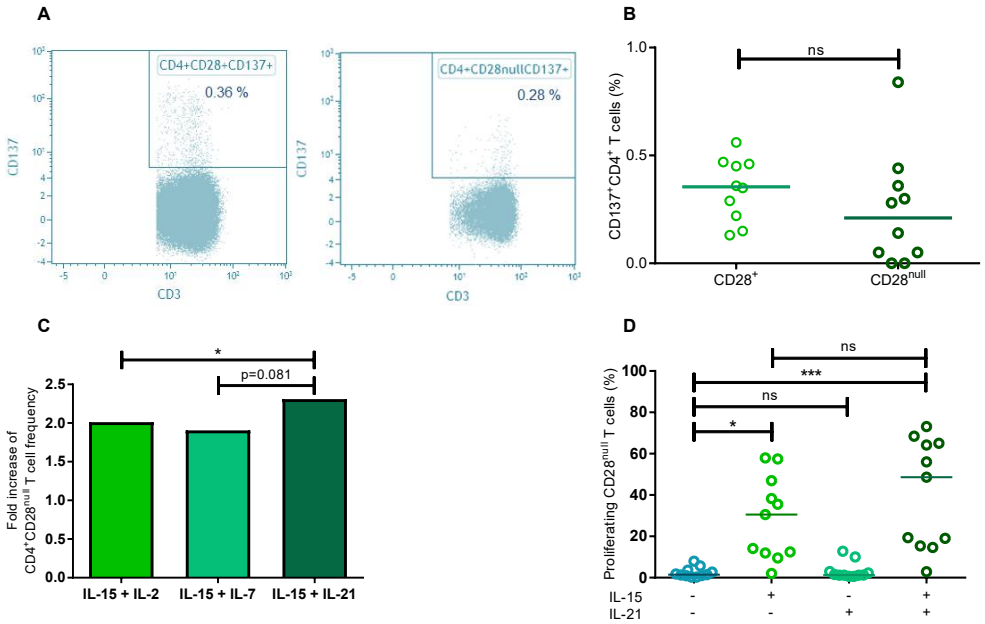

Figure 2. CD137 expression and effect of cytokines. (A) An example of CD137 expression by $CD4^+CD28^+$ T cells (dot plot on the left) and by $CD4^+CD28^{null}$ T cells (dot plot on the right) upon allogeneic stimulation is shown. (B) The frequency of CD137 expressing $CD4^+CD28^+$ and $CD4^+CD28^{null}$ T cells from 10 patients are shown after allogeneic stimulation. (C) The expansion of $CD4^+CD28^{null}$ T cells is shown in response to IL-15 + IL-2, IL-15 + IL-7 and IL-15 + IL-21 at 50 ng/ml (n=5). The Y-axis shows the expansion of $CD4^+CD28^{null}$ T cells, which is represented as the fold increase of the frequency of these cells. (D) The effect of cytokines on $CD4^+CD28^{null}$ T cells with regard to proliferation is analyzed in four different allogeneic conditions. The four allogeneic conditions are as follows; without cytokines, with IL-15, with IL-21 and with IL-15 + IL-21. Nine patients were stimulated with 2 different allogeneic stimulators, but due to lack of material, 11 samples could be analyzed with a non-parametric repeated measures ANOVA (Friedman test) between the different allogeneic conditions.

CD107a expression was higher in the non-proliferating fraction ($p<0.05$). Presence of both cytokines tended to show a higher frequency of CD107a-expressing cells within the proliferating fraction compared with the non-proliferating fraction ($p=0.088$). But compared with the allogeneic condition without cytokines, frequency of proliferating cells expressing CD107a increased significantly when IL-15 and IL-21 were added ($p<0.001$).

Figure 3C & 3D show that the cytotoxic molecules granzyme B and perforin are present within $CD4^+CD28^{null}$ T cells in the unstimulated condition. Overall, frequency of granzyme B and perforin positive $CD4^+CD28^{null}$ T cells did not increase significantly after allogeneic stimulation in presence of IL-15 and IL-21 (Figure 3C-D). However, frequency of granzyme B and perforin positive cells was mainly within the non-proliferating fraction of $CD4^+CD28^{null}$ T cells, whether or not the cells were cultured in presence of IL-15/IL-21 ($p<0.01$) (Figure 3E-F).

In summary, CD107a expression can be upregulated by IL-15 and IL-21. Next to this, there is no significant upregulation of the frequency of granzyme B^+ and $perforin^+$ $CD4^+CD28^{null}$ T cells in the proliferated cell fraction

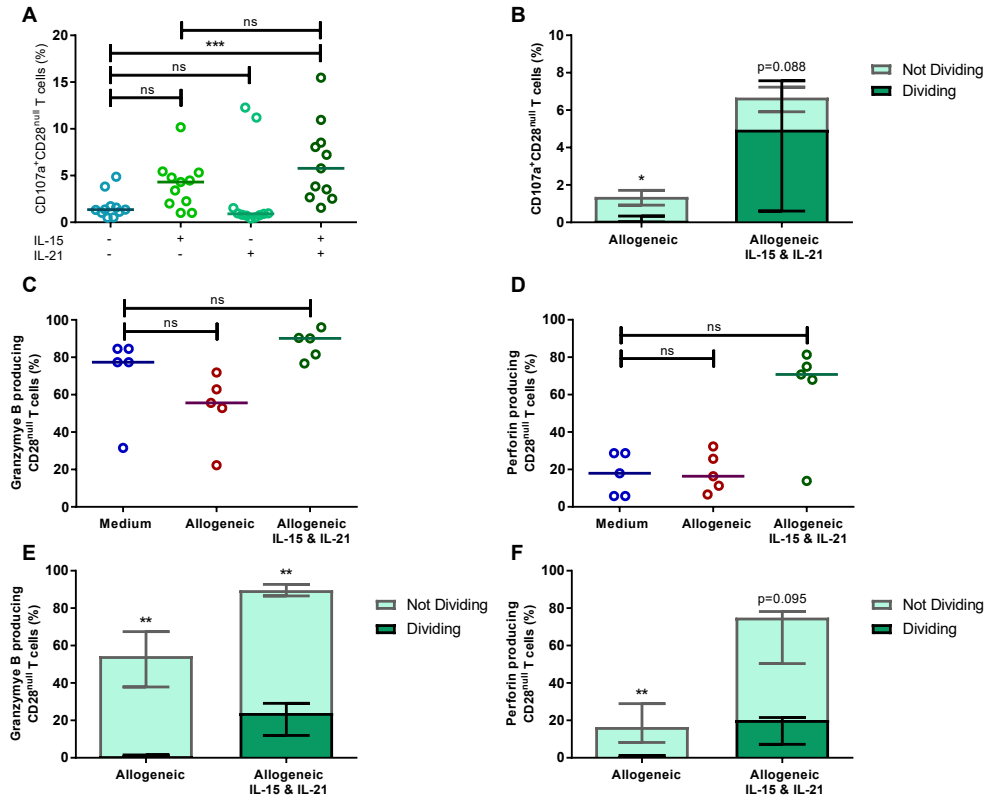

Figure 3. Degranulation and presence of cytotoxic molecules. (A) The effect of cytokines on $CD4^+CD28^{null}$ T cells with regard to CD107a expression is analyzed in four different allogeneic conditions. Six patients were stimulated with 2 different allogeneic stimulators in most of the cases, which led to n=11 for a non-parametric repeated measures ANOVA (Friedman test) between the different allogeneic conditions. (B) The differences between the frequencies of dividing and not-dividing $CD4^+CD28^{null}$ T cells with regard to CD107a expression are shown under two allogeneic conditions; allogeneic stimulation and allogeneic stimulation in combination with IL-15 and IL-21. (C) Frequencies of granzyme B and (D) perforin positive $CD4^+CD28^{null}$ T cells are shown between three different conditions (i.e. medium, allogeneic stimulation and allogeneic stimulation in combination with IL-15 and IL-21). Three patients were stimulated with 2 different allogeneic stimulators separately in most of the cases, which led to n=5 for a non-parametric repeated measures ANOVA (Friedman test) between the different conditions. The differences between the frequencies of dividing and not-dividing $CD4^+CD28^{null}$ T cells with regard to (E) granzyme B positivity and (F) perforin positivity are shown under two allogeneic conditions (i.e. allogeneic stimulation and allogeneic stimulation in combination with IL-15 and IL-21). Frequencies of cells are depicted as either individual percentages with medians or bars with medians. Significant differences were calculated and shown (ns: not significant, *$p<0.05$, **$p\leq0.01$, ***$p\leq0.001$).

Cytokine production is enhanced by IL-15 and IL-21 in CD4+CD28null T cells

The concentration of IFN-γ present in the supernatant was similar for cell culture conditions in which CD4+CD28null T cells were co-cultured with or without allogeneic stimulator cells (Figure 4A). Only with addition of IL-15 and IL-21, production of IFN-γ by CD4+CD28null T cells significantly increased from 0.87 to 6.32 pg/ml ($p<0.001$) (Figure 4B).

Same was also true for the concentration of TNF-α. Again, no difference was observed in the concentration of TNF-α between the unstimulated and allogeneic condition (Figure 4C). The concentration of TNF-α also showed a significant increase under influence of IL-15 (with/without IL-21) in CD4+CD28null T cells ($p<0.05$ for both conditions) (Figure 4D). Median TNF-α concentration increased from 0.54 (0.46–0.70) to 1.45 pg/ml (1.13–3.33) under influence of IL-15, and to 1.28 pg/ml (1.22–3.70) under influence of both cytokines.

In summary, cytokine production by CD4+CD28null T cells can, to a certain degree, be upregulated with IL-15 and IL-21.

CD4+CD28null T cells do not have suppressive properties

Directly upon isolation, CD4+CD28null T cells did not have suppressor activity on proliferating alloreactive T cells (data not shown). Analyzing the suppressive properties of allogeneic-expanded CD4+CD28null T cells, showed no significant suppression of allogeneic-induced proliferation by T cells depleted for CD4+CD28null T cells (data not shown). The suppressive potential of proliferating allogeneic CD4+CD28null T cells was addressed specifically for both cell populations. The results showed that sorted proliferating and non-proliferating CD4+CD28null T cells after allogeneic expansion were not displaying differences in suppressor activity (data not shown).

DISCUSSION

This study is the first in which pure fractions of CD4+CD28null T cells were used to investigate alloreactivity with/without exogenous cytokines. It is known that these cells can be drastically expanded and that they can comprise as much as 65% of the total CD4+ T-cell population in ESRD patients (30). CD4+CD28null T cells have also been shown to be associated with a higher as well as a decreased risk for rejection after RT (29, 34, 36). However until now, studies looking into the alloreactive potential of these cells are lacking.

Our results demonstrated that CD4+CD28null T cells showed no proliferation upon allogeneic stimulation alone. This was not due to the sorting procedure, since it did not influence CD28 expression. Stability of the CD28 phenotype was also observed by a study of Alonso-Arias et al. (94). Next to this, even within unsorted cells, CD4+CD28null T cells showed no proliferation in response to allogeneic stimulation contrary to CD4+CD28+ T cells. This means that, lack of responsiveness to allogeneic stimulation is a trait of these CD4+CD28null T cells. This could be due to the highly differentiated state of these cells. Continuous antigenic stimulation, by for example chronic viral antigens, can lead to

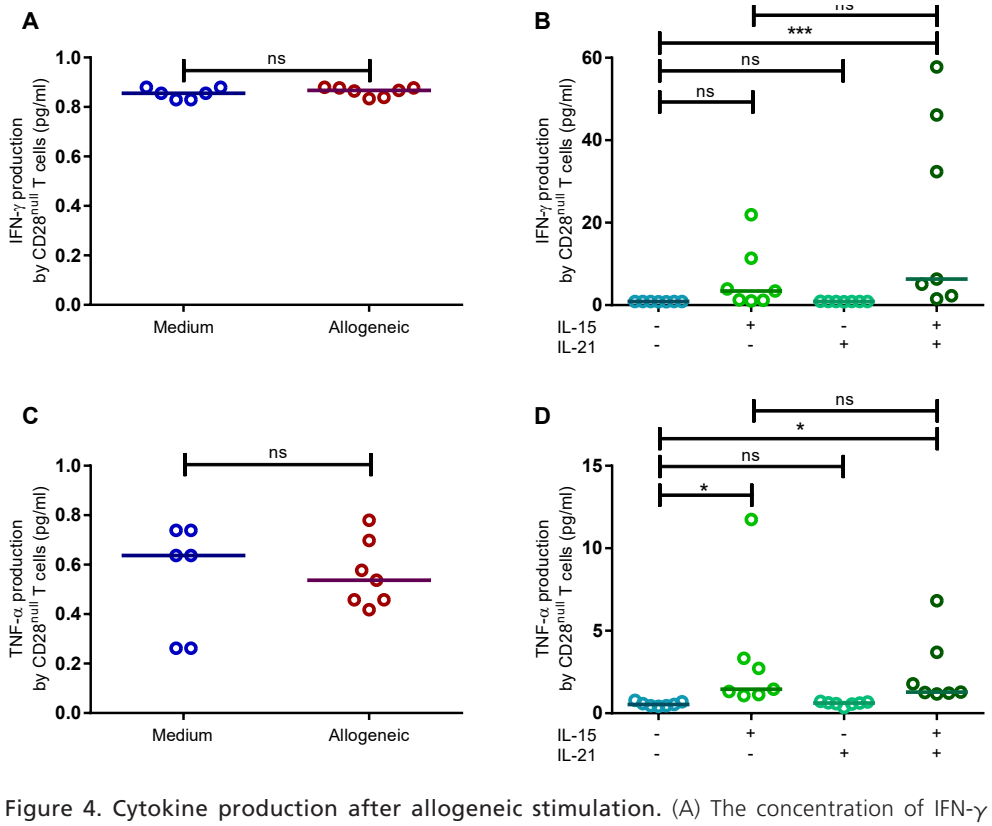

Figure 4. Cytokine production after allogeneic stimulation. (A) The concentration of IFN-γ production (pg/ml) by CD4+CD28null T cells are compared between negative control (medium) and allogeneic stimulation alone. (B) The effect of cytokines on CD4+CD28null T cells with regard to IFN-γ production is analyzed in four different allogeneic conditions. Four patients were stimulated with 2 different allogeneic stimulators separately in most of the cases, which led to *n*=6 for a paired analysis between the negative control and allogeneic condition (A) and *n*=7 for a non-parametric repeated measures ANOVA (Friedman test) between the different allogeneic conditions (B). (C) The concentration of TNF-α production (pg/ml) by CD4+CD28null T cells are compared between negative control (medium) and allogeneic stimulation alone. (D) The effect of cytokines on CD4+CD28null T cells with regard to TNF-α production is analyzed in four different allogeneic conditions. The same sample size and statistical analyses apply also to these figures. Frequencies of cells are depicted as individual percentages as well as medians. Significant differences were calculated and shown (ns: not significant, *$p<0.05$, **$p\leq0.01$, ***$p\leq0.001$).

differentiation and expansion of cells directed against specific antigens (42, 43). Therefore, the provided antigen might differ in strength of stimulation for these cells to respond properly. This is further supported by our finding that CMV-antigen was sufficient enough for CD4+CD28null T cells to proliferate significantly compared with an allogeneic stimulus.

However, alloantigen specific (CD137+)CD4+CD28null T cells did exist, but needed additional stimulation to become responsive. We found that proliferation upon allogeneic stimulation could be induced by IL-15. This is especially important as renal tubular epithelial cells, next to cells of the immune system, can also produce IL-15 (97, 98). Furthermore,

IL-15 is also required for survival of CD4+CD28null T cells in the periphery (99, 100). IL-15 did not upregulate the presence of granzyme B and perforin in CD4+CD28null T cells significantly, but positivity for cytotoxic molecules was mostly within the non-proliferating fraction, suggesting the release of these molecules by the proliferating fraction. This is further supported by our finding that, in presence of IL-15 and IL-21, CD107a+ proliferating CD4+CD28null T cells significantly increased in frequency. This means that IL-15 and IL-21 could optimize the cytotoxic potential of these cells towards the renal allograft, especially when the significant amounts of granzyme B and perforin within these cells are taken into account. Presence of these intracellular cytotoxic molecules within the unstimulated condition was also shown by us, which is in accordance with previous studies (23, 29, 30, 32). Next to this, we also observed that cytokine production by CD4+CD28null T cells was limited upon allogeneic stimulation, but that addition of exogenous IL-15 and IL-21 enhanced IFN-γ and TNF-α production. This supports the finding that allogeneic stimulation does not provide sufficient stimulation for CD4+CD28null T cells to produce cytokines unless supported by IL-15 together with IL-21.

Next to their reduced alloreactive potential, another mechanism contributing to their association with reduced risk for rejection, might involve suppressive properties. Several studies have shown that within CD8+ T cells, suppressive/regulatory cells are present that are more of an effector type and thus can lack CD28 (101-103). Since CD4+CD28null T cells resemble CD8+CD28null T cells, they might show similarities with regard to suppressive/regulatory function. However, our results showed that these properties were not present in CD4+CD28null T cells. Even a functional division of CD4+CD28null T cells into dividing and non-dividing cells upon allogeneic stimulation, did not show any differences.

These mechanisms together suggest that these cells could be harmful for the renal allograft, if the optimal conditions are met. Whether these cells can be called exhausted is a matter of debate, since they are still able to proliferate and exert cytotoxic functions in multiple ways (94, 95). Their function seems also to be stimulus-dependent. For example, we showed that these cells were able to proliferate upon stimulation with CMV-antigen. This means that these cells are not exhausted and still harbor significant proliferative potential. But when an allogeneic stimulation was applied, no significant proliferation could be observed unless the latter was promoted by especially IL-15. This difference might be caused by the increased specificity of these cells for CMV, as discussed earlier. Infection with CMV can lead to expansion of T cells with an oligoclonal repertoire (104, 105). This diminished T-cell receptor repertoire diversity might impair the ability to recognize new antigens. Furthermore, it could be that the affinity/avidity of these T cells to alloantigen might not be as high as to CMV-antigen, and dependent on IL-15 and IL-21 to respond. The additional requirement of IL-15 and in lesser extent of IL-21 in the setting of alloreactivity, might also explain the improved renal allograft survival and graft function in patients with co-stimulatory blockade therapy belatacept, compared with cyclosporine based regimens (106). Inhibition of the co-stimulatory pathway leads to anergy of CD28+ T cells by binding of CD80/86 on an antigen presenting cell, which on its turn usually

binds to CD28 on T cells (107). Since CD4+CD28null T cells need additional cytokines next to allogeneic stimulation, inhibition of immune cells that can provide these cytokines will lead to a low alloresponsive state. In co-stimulatory blockade resistant rejections however, the severity of rejections are more serious compared with rejections under cyclosporine (108, 109). A study by de Graav et al. demonstrated the presence of CD86+ monocytes in the rejected allograft under belatacept treatment (109). This severity could be explained by the production of IL-15 by these monocytes and probably also the renal tubular epithelial cells, which might lead to activation of highly proinflammatory and cytotoxic CD28null T cells.

In conclusion, CD4+CD28null T cells can become alloreactive when the proper conditions are met, especially in presence of IL-15. IL-21 can enhance the cytotoxic potential of CD4+CD28null T cells.

DISCLOSURES

The authors declare no competing financial and commercial interests. This study was funded by the Dutch Kidney Foundation (KSPB.10.12).

Chapter 4

UREMIA-ASSOCIATED PREMATURE AGEING OF T CELLS DOES NOT PREDICT INFECTIOUS COMPLICATIONS AFTER RENAL TRANSPLANTATION

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ABSTRACT

Background

Patients with end-stage renal disease have prematurely aged T cell systems. We tested whether T-cell ageing parameters were associated with the risk of infections after renal transplantation (RTx).

Methods

We studied 188 patients over 1 year. Peripheral T cells were analyzed before and at 3 and 6 mo after RTx for frequency of recent thymic emigrants, relative telomere length and differentiation status. These parameters were related to the occurrence of opportunistic and serious infections.

Results

Overall, 84 patients developed an infection. In this group, 50 developed an opportunistic infection and 53 developed a serious infection. T-cell ageing parameters assessed before RTx were not associated with infection risk. The memory T cells showed a decrease within the first 3 months in both groups ($p<0.001$). The $CD4^+$ memory T cells increased between 3 and 6 months within the infection group ($p=0.015$). The number of $CD8^+$ memory T cells increased in both groups ($p<0.001$) but reached baseline levels only in the infection group. In the infection group, the $CD8^+CD28^{null}$ T-cell percentage increased between 3 and 6 months ($p=0.024$), tending to be higher than at baseline ($p=0.061$). These differences in post-RTx dynamics resulted from infections.

Conclusion

Parameters of uremia-associated premature ageing of peripheral T cells do not predict posttransplant infections.

INTRODUCTION

Patients with end-stage renal disease (ESRD) have a prematurely aged T-cell system. This process is likely induced by the pro-inflammatory uremic environment created by the loss of renal function (54) and is marked by lower thymic output, increased T-cell differentiation and telomere shortening (11).

The changes in the peripheral T-cell compartment of ESRD patients resemble the physiological changes in the immune systems of healthy elderly persons (13, 20, 58). Studies analyzing these T-cell ageing parameters in ESRD patients, using healthy elderly persons as a reference, showed a discrepancy of 15–20 years between the immunological and chronological ages of T cells of ESRD patients (11, 12).

A characteristic of this process is lower thymic output, noted by a decrease in newly formed naive T cells (i.e. recent thymic emigrants [RTE]) (11). In addition, increased T-cell differentiation leads to a more differentiated memory T-cell compartment. Both mechanisms together reduce the percentage of naive T cells in the circulation to a relatively high extent (11, 56). Another feature of T-cell differentiation is the loss of the costimulatory molecule CD28 on the surface of memory T cells (27), enabling these cells to become activated without costimulation. Furthermore, the increments of proliferation and differentiation reduce the telomere length (11, 30, 57).

The prematurely aged T-cell system of ESRD patients is associated with a reduced vaccination response (8-10, 59) and an increased susceptibility to infection (7). The latter is a major cause of mortality in ESRD patients, in addition to cardiovascular disease (7, 110, 111). Immunosuppressive drugs given after renal transplantation (RTx) increase the risk of severe and opportunistic infections (OIs). Whether the degree of preexistent uremia-associated immunodeficiency adds to this increased susceptibility is unknown. In healthy elderly persons, an immunological risk profile (IRP) has been established (20, 112). Potentially, the identification of an IRP based on ageing parameters in patients eligible for RTx would allow for a more personalized regimen of immunosuppressive medication.

In this study, we investigated whether the degree of premature T-cell ageing prior to and within the first year after RTx is associated with the risk of infections after RTx.

MATERIALS AND METHODS

Study population

All ESRD patients who received a kidney from a living donor in the period from November 1, 2010, to October 1, 2013, were considered for participation. We excluded patients if they were lost to follow-up ($n=6$) or received T-cell depletion therapy ($n=17$). The latter were excluded because these agents cause significant long-lasting T-cell depletion with a subsequent increased risk of severe infections (113, 114). All patients gave written informed consent to participate in the clinical trial and in the substudy, which is presented in this paper. The study was approved by the medical ethics committee of the Erasmus Medical Center (MEC number 2010-080, EudraCT 2010-018917-30) and was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

All patients received similar immunosuppressive regimens. This included induction therapy with basiliximab (Simulect; Novartis, Basel, Switzerland) and maintenance therapy with tacrolimus (Prograf; Astellas Pharma, Tokyo, Japan), mycophenolate mofetil (Cellcept; Roche, Basel, Switzerland) and glucocorticoids.

Clinical variables were assessed, as shown in Table 1, including age, sex, cytomegalovirus (CMV) seropositivity, HLA mismatches, current and historical panel reactive antibody scores, number of previous RTx, warm ischemia time, underlying cause of renal failure, preemptive RTx and genetically related RTx. HLA typing was performed according to international standards (American Society for Histocompatibility and Immunogenetics, European Federation for Immunogenetics) using serological and DNA-based techniques. The panel reactive antibodies were determined at the laboratory of the blood bank in Leiden, the Netherlands.

The infections occurring within the first year after RTx were scored for type of infection (serious infection [SI] or OI), the causative microorganism (viruses, bacteria, fungi), the number of infections per patient and the time until infection development (in days) (Table 2). We defined an OI as an infection associated with cellular and humoral immunosuppression. These included viral infections such as CMV, Epstein-Barr virus (EBV), herpes simplex virus, varicella zoster virus and polyomavirus; bacterial infections such as typical and atypical mycobacteria, Listeria, Legionella and Nocardia; and fungal infections such as Pneumocystis jirovecii pneumonia (PCP), Aspergillus, Cryptococcus and Candida. CMV viremia was detected by viral genome quantification in blood by real-time polymerase chain reaction (PCR), and CMV disease was considered if this was accompanied by clinical signs and/or organ involvement. Quantification of EBV, varicella zoster virus, herpes simplex virus and polyomavirus DNA was assessed by real-time PCR assays. EBV infection was considered if there were clinical signs and/or organ involvement. BK nephropathy was diagnosed if histologically confirmed via a renal biopsy. We defined an SI as any infection requiring admission to the hospital. We excluded infections of iatrogenic cause, such as urinary tract infections due to catheterization. These SIs were categorized into four groups based on the tract that was involved (i.e. urinary tract, respiratory tract, gastrointestinal, and other) (Table 2).

Isolation of peripheral blood mononuclear cells

By using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), peripheral blood mononuclear cells were isolated from heparinized blood samples. Blood was drawn from RTx recipients the day before RTx (T=0), and at 3 months (T=3) and 6 months (T=6) after RTx. The isolated peripheral blood mononuclear cells were stored at -150°C with a minimum amount of 10×10^6 cells per vial.

T-cell differentiation status and absolute numbers of T-cell subsets

To determine T-cell differentiation status, whole-blood staining was performed based on a study by Sallusto et al. (22), as described in detail in a previous study (11). The absolute numbers of the different T-cell subsets were determined via Trucount staining (Becton Dickinson, Franklin Lakes, NJ), as described by Bouvy et al. (115).

Relative telomere length

To determine the relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells, flow fluorescent in situ hybridization was performed, as described in detail by a previous study (11).

Statistical analysis

All variables were presented as medians with interquartile ranges. The differences between continuous variables were assessed with the Mann–Whitney U test. The difference between categorical variables was analyzed with either the Pearson chi-square test or the Fisher exact test, depending on the expected values in any of the cells of a contingency table. In case of repeated measurements, a nonparametric repeated measures analysis of variance (Friedman test) was used, and a correction was made for multiple comparisons. When there was a significant difference between the two patient groups, a Cox regression analysis was used to assess whether an association could be observed between a variable and the development of infection after RTx. The significance level (p-value) was two-tailed, and an α of 0.05 was used for all analyses. Statistical analyses were performed using SPSS version 21.0 for Windows (IBM Corp., Armonk, NY) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Figures were created with GraphPad Prism 5.

RESULTS

Patient and infection characteristics

Of the 211 eligible patients, six were lost to follow-up. Four of those patients underwent a transplantectomy due to therapy-resistant acute rejection or vascular problems shortly after RTx; the other two patients were referred to their own hospitals soon after RTx. Of the remaining 205 patients, 17 received T-cell depletion therapy. This led to 188 patients for further analysis.

Patient characteristics are shown in Table 1. The median patient age was 57 years, and the median donor age was 53 years. The majority of patients (93%) underwent transplantation for the first time, 13 patients (7%) were transplanted for the second time, and one patient (1%) was transplanted for a third time. Of all analyzed patients, 84 (45%) developed an infection within the first year after RTx. The CMV donor/recipient (D/R) serostatus was significantly more often a D+/R– configuration in the infection group (p=0.009). Furthermore, a D–/R+ configuration was more often observed in the group without infection (p=0.027). There were no other differences between the groups.

Characteristics of the infections are shown in Table 2. Fifty patients developed an OI, and 53 developed an SI. The median time for development of an infection was 2.9 months to the first OI and 3.3 months to the first SI; the overall median time for development of an infection was 2.9 months. The majority of infections (85%) occurred within the first 6

Table 1. Patient characteristics

RT Patients (n=188)	No Infection (n=104) (55%)	Infection (n=84) (45%)	P
Age recipient	57 (48 – 63)	57 (46 – 65)	0.522
Age Donor	53 (43 – 61)	55 (39 – 63)	0.647
Male recipient	65 (63%)	50 (60%)	0.677
Male donor	50 (48%)	44 (52%)	0.557
CMV seropositivity recipient	67 (64%)	45 (54%)	0.132
CMV serostatus D/R			
-/-	26 (25%)	18 (21%)	0.565
-/+	24 (23%)	9 (7%)	0.027
+/-	11 (11%)	21 (25%)	0.009
+/+	43 (41%)	36 (43%)	0.835
Mismatch HLA class I	2 (2 – 3)	2 (2 – 3)	0.622
Mismatch HLA class II	1 (1 – 2)	1 (1 – 2)	0.377
Mismatch HLA class I and II	4 (3 – 5)	4 (3 – 5)	0.924
Current PRA (%)	0 (0 – 4)	0 (0 – 4)	0.887
Historical PRA (%)	4 (0 – 4)	4 (0 – 5)	0.589
Amount of RTx	1 (1 – 1)	1 (1 – 1)	0.072
Warm ischemia time	20 (17 – 24)	19 (16 – 23)	0.177
Underlying disease			
Nephrosclerosis/atherosclerosis/ hypertension	26 (25%)	19 (23%)	0.704
Primary glomerulopathies	15 (14%)	11 (13%)	0.793
Diabetes	18 (17%)	21 (25%)	0.196
Urinary tract infections/stones	3 (3%)	2 (2%)	>0.999
Reflux nephropathy	7 (7%)	2 (2%)	0.303
Polycystic Kidney Disease	17 (16%)	17 (20%)	0.491
Other	12 (12%)	8 (10%)	0.656
Unknown	6 (6%)	4 (5%)	>0.999
Pre-emptive RTx	49 (47%)	30 (36%)	0.115
Genetically-related RTx	42 (40%)	32 (38%)	0.749

Data are presented as median (interquartile range) except as noted. Values in bold represent significant p-values (p<0.05). CMV, cytomegalovirus; D/R, donor/recipient; PRA, panel reactive antibody; RTx, renal transplantation.

months after RTx, and within this period, the majority (66%) occurred within the first 3 months (Figure 1A). Most of the OIs were of viral origin, accounting for 96% of the cases. Approximately half of the SIs (49%) were urinary tract infections (Table 2 and Figure 1B).

Pretransplant T-cell ageing parameters

Prior to RTx, there was no difference in differentiation status or in numbers of $CD4^+$ and $CD8^+$ T cells or the various subsets between the groups with and without infection

Table 2. Infection characteristics

Opportunistic Infections	50 (60%)
Viral infections	48 (96%)
CMV viremia	13 (27%)
CMV disease	12 (25%)
BK viremia	17 (35%)
BK nephropathy	2 (4%)
EBV viremia	6 (13%)
EBV infection	2 (4%)
Herpes Simplex	1 (2%)
Varicella Zoster	3 (6%)
Bacterial Infections	0 (0%)
Fungal Infections	3 (6%)
Candida	3 (100%)
PCP pneumonia	0 (0%)
Number of opportunistic infections per patient	1 (1 – 1)
Days until first opportunistic infection	88 (70 – 185)
Serious Infections	53 (63%)
Viral Infections	9 (32%)[1]
Bacterial Infections	38 (91%)[1]
Fungal infections	1 (4%)[1]
Type of serious infections	
Urinary tract infections	26 (49%)
Respiratory tract infections	12 (23%)
Gastrointestinal infections	18 (34%)
Other infections	15 (28%)
Number of serious infections per patient	2 (1 – 2)
Days until first serious infection	99 (50 – 134)
Days until first infection	87 (55 – 140)

Data are presented as absolute number (percentage) or as median (interquartile range). CMV, cytomegalovirus; EBV, Epstein-Barr virus; PCP, Pneumocystis jirovecii pneumonia. [1]The percentages were calculated via exclusion of missing values and refer to the 53 patients with a serious infection.

(Table 3). Moreover, the RTLs in both T-cell compartments were not discriminative between groups (Table 3). In addition, similar frequencies of RTE were detectable in both groups before transplant (Table 3).

Because a primary CMV infection has a significant impact on the T-cell differentiation status (27, 42) and more D+/R– RTxs were present in the infection group, we performed the same analyses without the D+/R– configuration. These analyses also showed no significant differences between groups (data not shown). Moreover, performing the same analyses without CMV viremia/disease showed no differences between groups (Table S1). Finally, we focused on the development of CMV viremia/disease separately. We divided these patients into two different risk groups. The D+/R+ and D–/R+ configurations were

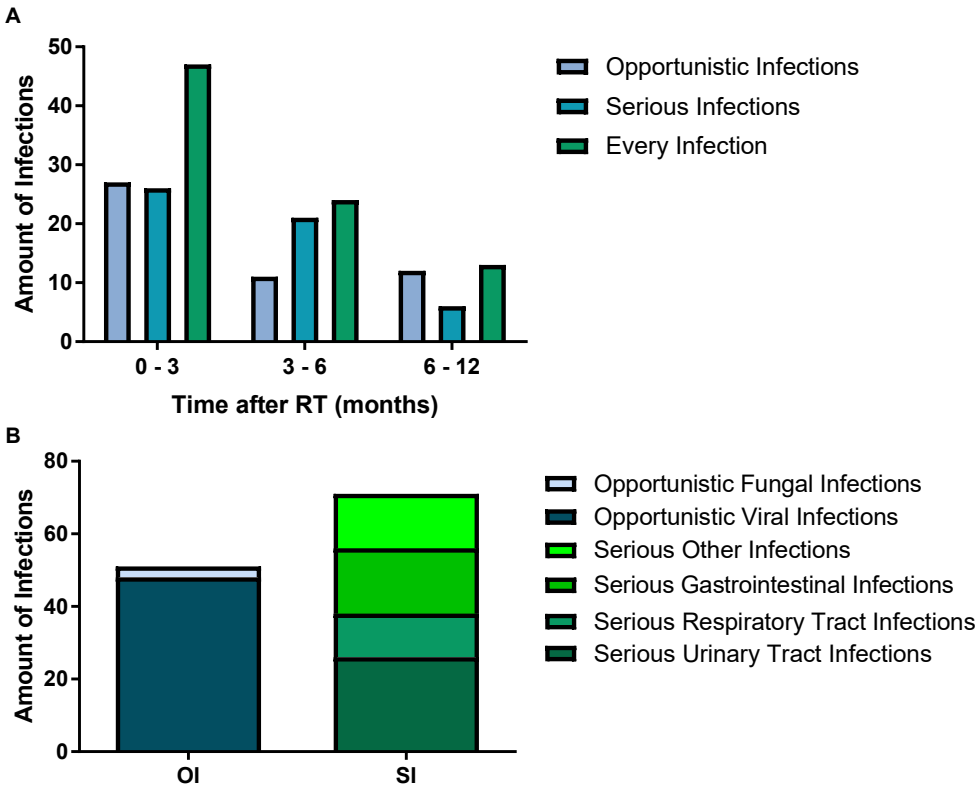

Figure 1. Infection characteristics. (A) The amount of infections are represented in three groups at RTx and at 3 and 6 months after RTx. The light blue bar represents the number of OIs, the blue bar represents the number of SIs, and the green bar represents every infection. (B) The type of infections are presented. Among the OIs, the dark blue bar represents the number of viral OIs and the light blue bar represents the number of fungal OIs. Among the SIs, from dark green to light green, the bars represent the number of urinary tract infections, the number of respiratory tract infections, the number of gastrointestinal infections, and the number of other infections, respectively. OI, opportunistic infection; RTx, renal transplantation; SI, serious infection.

defined as moderate-risk groups. From the 112 patients with these configurations, 67 patients did not develop an infection and 8 developed a CMV viremia/disease. When we analyzed the differences between these two groups with regard to $CD28^{null}$ T cells, RTL and RTE, we did not observe any differences prior to RTx (Table S2). Patients with the D+/R– configuration were defined as high risk (*n*=32). Of these patients, 16 developed CMV viremia/disease and 11 did not. The only difference we observed between these two groups was, that the percentage of $CD8^{+}CD28^{null}$ T cells was higher in the group without infection (Table S3). Furthermore, a negative correlation between the frequency of $CD8^{+}CD28^{null}$ T cells and the development of CMV viremia/disease (ρ= –0.424, p=0.031) was observed, and a linear regression analysis showed a similar result (B= –0.016, p=0.031). We then performed a Cox regression analysis to assess whether higher frequencies of these cells were associated with a lower risk of development of CMV viremia/disease. We defined time as days until the development of CMV viremia/disease. This analysis showed that the hazard ratio per percentage increase of $CD8^{+}CD28^{null}$ T cells was equal to 0.95 (95% confidence interval 0.895–0.997, p=0.038). These results suggest that in patients with a D+/R– configuration, a higher frequency of $CD8^{+}CD28^{null}$ T cells is associated with a lower risk of development of CMV viremia/disease.

Seventeen patients developed an acute rejection, for which they received 1000 mg methylprednisolone intravenously on 3 days consecutively. These patients were equally present within both groups (data not shown).

Immunological risk profile

Several studies have tried to assess an IRP in persons with immunological ageing (20, 112). In these studies, an inverted CD4/CD8 ratio was associated with poor survival (20, 112). An important contributor to this inverted ratio was CMV seropositivity (21, 58). Based on these findings, we assessed whether an inverted CD4/CD8 ratio and CMV seropositivity were able to discriminate between groups. Furthermore, having both of these characteristics was defined as a high-risk profile. Having only one or neither of these characteristics was defined as a low-risk profile. The results showed that none of these variables were able to predict the risk of an infection after RTx (Table 4), and none were able to discriminate between OIs and SIs (data not shown).

Posttransplant $CD4^{+}$ T-cell subset dynamics

The absolute CD4+ T-cell numbers declined significantly between T=0 and T=3 ($p < 0.01$) in both groups (Figure 2A). The $CD4^{+}$ naive T-cell numbers were not affected after RTx in both groups (Figure 2B), but the absolute $CD4^{+}$ memory T-cell numbers declined significantly between T=0 and T=3 ($p < 0.001$ for both groups) (Figure 2C). These numbers increased again between T=3 and T=6 and were significant only for the infection group (p=0.015). $CD4^{+}CD28^{null}$ T-cell frequencies were not different after RTx between groups (Figure 2F). Exclusion of the D+/R– group for CMV did not affect the observed dynamics of the $CD4^{+}$ T-cell compartment.

The different $CD4^{+}$ T-cell subsets at T=3 were not associated with the development of an infection after this period (data not shown). Given the small number of infections after 6 months (only 13 patients with an infection), T=6 was not considered as a time point for the prediction of infections thereafter in the $CD4^{+}$ T-cell subsets. In contrast, exclusion of CMV viremia/disease showed some differences. The $CD4^{+}$ memory T cells, $CD8^{+}$ T cells and the $CD8^{+}$ naive T cells seemed lower in the infection group (p=0.078, p=0.031 and p=0.053 respectively) (Table S4); however, further analyses showed that there were no correlations between these variables and the development of infections after 3 months (Table S5).

Table 3. T-cell ageing parameters prior to renal transplantation

At T=0 (n=188)	No Infection (n=104) (55%)	Infection (n=84) (45%)	P
CD4 cells (cells/μL)	688 (524 – 887)	691 (441 – 913)	0.763
CD4 naïve (cells/μL)	207 (102 – 336)	170 (96 – 326)	0.642
CD4 memory (cells/μL)	467 (357 – 573)	456 (296 – 650)	0.895
Naïve/memory CD4	0.45 (0.23 – 0.70)	0.40 (0.21 – 0.73)	0.916
CD4 CM of CD4 memory (%)	61.7 (49.8 – 73.5)	60.6 (46.4 – 68.8)	0.476
CD4 EM of CD4 memory (%)	35.4 (24.5 – 45.8)	36.3 (28.5 – 47.8)	0.359
CD4 EMRA of CD4 memory (%)	1.9 (0.7 – 4.2)	2.1 (1.3 – 4.1)	0.305
CD28null of CD4 (%)	3.0 (0.5 – 8.9)	2.5 (0.3 – 10.9)	0.681
CD8 cells (cells/μL)	390 (254 – 503)	328 (210 – 496)	0.122
CD8 naïve (cells/μL)	64 (32 – 104)	53 (25 – 105)	0.433
CD8 memory (cells/μL)	283 (189 – 405)	251 (136 – 345)	0.129
Naïve/memory CD8	0.20 (0.10 – 0.53)	0.24 (0.10 – 0.54)	0.689
CD8 CM of CD8 memory (%)	6.3 (3.5 – 13.5)	7.0 (2.7 – 12.0)	0.723
CD8 EM of CD8 memory (%)	45.1 (29.8 – 58.5)	47.1 (28.9 – 61.4)	0.710
CD8 EMRA of CD8 memory (%)	44.4 (27.8 – 64.9)	44.1 (26.4 – 58.7)	0.697
CD28null of CD8 (%)	43.4 (23.8 – 61.2)	37.0 (17.6 – 59.6)	0.269
RTL CD4	12.2 (9.8 – 14.7)	11.6 (8.9 – 15.1)	0.954
RTL CD8	11.5 (9.5 – 14.6)	11.0 (8.9 – 15.8)	0.997
CD31 of naive CD4 (%)	65.8 (55.8 – 75.4)	66.8 (52.9 – 72.8)	0.382
CD31 of naive CD8 (%)	97.6 (94.5 – 98.8)	97.9 (94.4 – 99.2)	0.346

Data are presented as medians (interquartile range). EMRA, effector memory CD45RA$^+$; RTL, relative telomere length; T=0, time of renal transplantation; TCM, central memory T cells; TEM, effector memory T cells.

Table 4. Immunological Risk Profile

RT Patients (n=188)	No Infection (n=104) (55%)	Infection (n=84) (45%)	P
CMV seropositivity			
CMV negative	37 (36%)	39 (46%)	0.132
CMV positive	67 (64%)	45 (54%)	
CD4/CD8 ratio			
≥ 1	90 (87%)	73 (87%)	0.923
< 1	13 (13%)	11 (13%)	
Immune Risk Profile			
Low Risk Profile	93 (89%)	77 (92%)	0.745
High Risk Profile	10 (10%)	7 (8%)	

Data are presented as absolute number (percentage). CMV, cytomegalovirus; RTx, renal transplantation.

Posttransplant CD8$^+$ T-cell subset dynamics

Absolute CD8$^+$ T-cell numbers declined significantly during the first 3 months in the groups without and with infection (p<0.001 and p=0.005, respectively) (Figure 3A). Between T=3 and T=6, CD8$^+$ T-cell numbers increased again (p=0.009 and p=0.007, respectively), approaching baseline levels at T=6. Naive T-cell numbers were not affected after RTx in both groups (Figure 3B). The CD8$^+$ memory T-cell numbers (Figure 3C) declined significantly during the first 3 months after RTx in both groups (p<0.001), with a significant increase thereafter (p<0.001), reaching baseline levels only in the infection group. For the group without infection, a shift was observed toward relatively more highly differentiated effector memory CD45RA$^+$ T cells at T=6 (Figure 3D–F). The CD8$^+$CD28^{null} T-cell frequencies declined significantly between T=0 and T=3 (p<0.001) in the group without infection (Figure 3G), with a subsequent increase thereafter (p=0.007). In the infection group, the CD8$^+$CD28^{null} T-cell frequencies increased significantly (p=0.024) between T=3 and T=6, almost resulting in a larger value than at baseline (p=0.061).

The different CD8$^+$ T-cell subsets at T=3 were not associated with the development of infections after this period (data not shown).

RTL and RTE after transplant

The RTL of the CD4$^+$ T-cell population was not affected after RTx in both groups (Figure 4A). The RTL of the CD8$^+$ T-cell population increased only in the infection group between T=0 and T=3 (p=0.018) and remained similar thereafter (Figure 4B). The RTL of the CD8$^+$ T-cell population in the group without infection did not change during the whole period (Figure 4B).

In the group without infection, the percentage of RTE within the naive CD4$^+$ T-cell population declined between T=0 and T=3 (p=0.013) (Figure 4C), whereas the percentage of RTE within the naive CD8$^+$ T-cell population showed no differences (Figure 4D). Frequencies of RTE remained similar during the rest of the period for naïve CD4$^+$ T cells. In the infection group, the percentage of RTE within the naive CD4$^+$ and CD8$^+$ T-cell population remained similar (Figures 4C and D).

Neither RTL nor the number of RTE at T=3 was associated with subsequent infections thereafter.

DISCUSSION

This prospective study tested the hypothesis that the degree of premature immunological ageing before and after transplant predicts the risk of infections after RTx. Furthermore, we compared our findings with immune risk parameters defined by other studies (20, 112). This study is the first in its field because of its prospective nature, clinical setting, well-defined parameters and large group of patients with the exclusion of those receiving T-cell depletion therapy.

The majority of infections occurred within the first 6 months after RTx, which is in accordance with the generally observed course of infections after solid organ

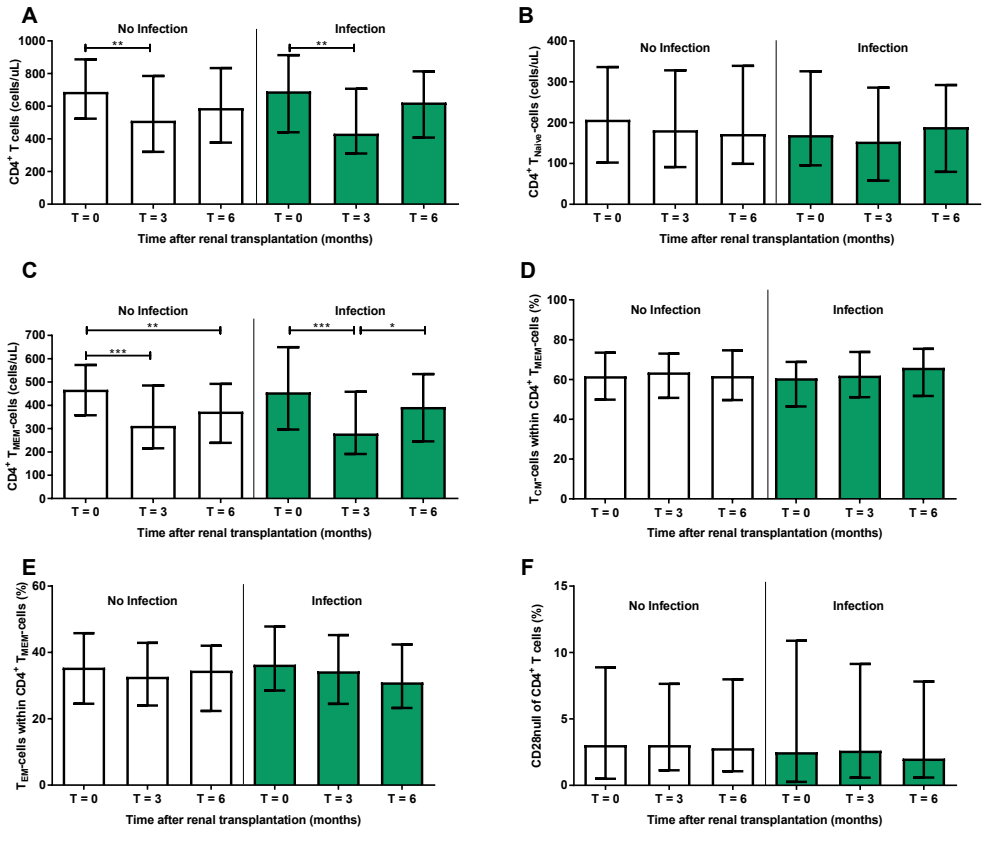

Figure 2. Dynamics of CD4⁺ T cells. The courses of (A) CD4⁺ T cells, (B) CD4⁺ T_{NAIVE}, (C) CD4⁺ T_{MEM}, (D) CD4⁺ T_{CM} within CD4⁺ T_{MEM}, (E) CD4⁺ T_{EM} within CD4⁺ T_{MEM}, and (F) CD4⁺$CD28^{null}$ within CD4⁺ T cells at T=0, T=3, and T=6 are represented. The white bars represent the no-infection group, and the green bars represent the infection group. The bars represent the medians with interquartile ranges. Absolute numbers of cells are depicted (cells/μL) for graphs (A–C) and percentages are depicted for graphs (D–F). Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001). RTx, renal transplantation; T=0, time of RTx; T=3, 3 months after RTx; T=6, 6 months after RTx; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{MEM}, memory T cells; T_{NAIVE}, naive T cells.

transplantation (116). The most common OI after RTx was CMV related, accounting for approximately half of these infections; this finding is supported by other studies (117-119). Urinary tract infections accounted for the majority of SIs in our study and have also been described by previous studies (120-122).

T-cell ageing parameters before transplant were not predictive for an infection, and exclusion of primary CMV infections (D+/R–) did not influence this. This indicates that the degree of T-cell ageing is not associated with the risk of infection after RTx in contrast to the association between aged T cells and a lowered risk for acute rejection (66, 67). The only association we found was between the development of CMV viremia/disease

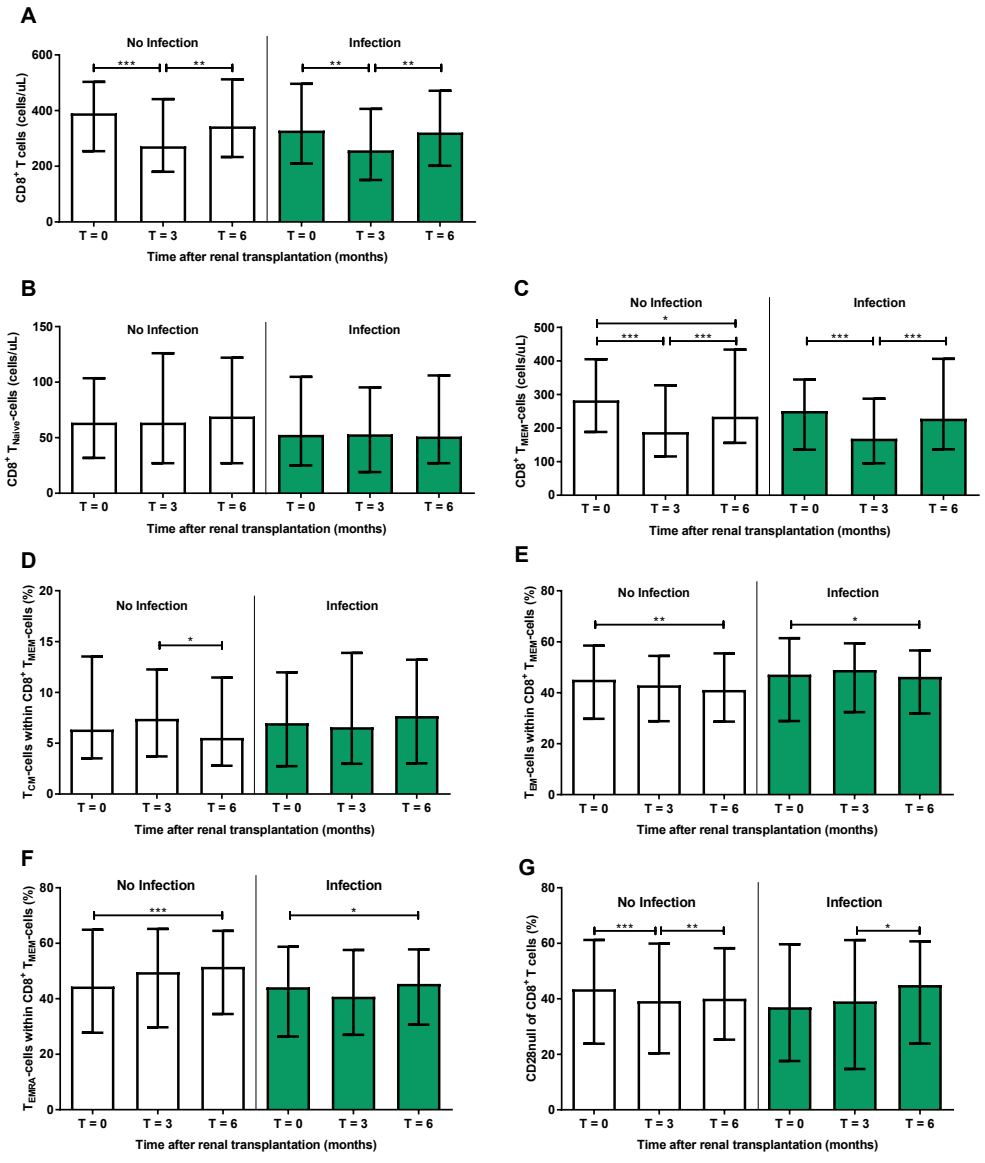

Figure 3. Dynamics of CD8⁺ T cells. The courses of (A) CD8⁺ T cells, (B) CD8⁺ T_{NAIVE}, (C) CD8⁺ T_{MEM}, (D) CD8⁺ T_{CM} within CD8⁺ T_{MEM}, (E) CD8⁺ T_{EM} within CD8⁺ T_{MEM}, (F) CD8⁺ EMRA T cells within CD8⁺ T_{MEM}, and (G) CD8⁺$CD28^{null}$ within CD8⁺ T cells at T=0, T=3, and T=6 are represented. The white bars represent the no-infection group, and the green bars represent the infection group. The bars represent the medians with interquartile ranges. Absolute numbers of cells are depicted (cells/μL) for graphs (A–C), and percentages are depicted for graphs (D–G). Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001). EMRA, effector memory CD45RA⁺; RTx, renal transplantation; T=0, time of RTx; T=3, 3 months after RTx; T=6, 6 months after RTx; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{MEM}, memory T cells; T_{NAIVE}, naive T cells.

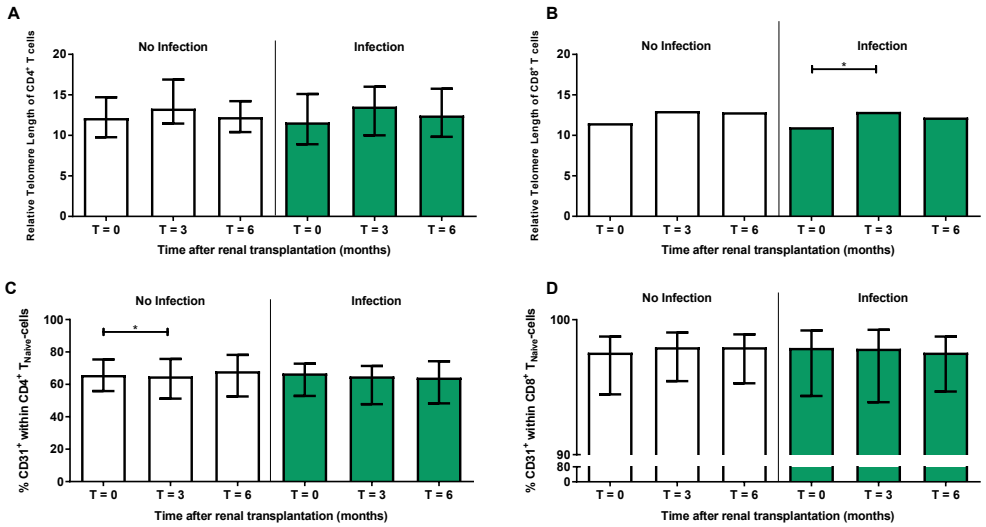

Figure 4. RTL and RTE after renal transplantation. The course of the RTL of (A) $CD4^+$ and (B) $CD8^+$ T cells and the course of RTE within (C) $CD4^+$ T_{NAIVE} and (D) $CD8^+$ T_{NAIVE} at T=0, T=3, and T=6 are represented. The white bars represent the no-infection group, and the green bars represent the infection group. The bars represent the medians with interquartile ranges. For the RTE, percentages of cells are depicted. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001). RTE, recent thymic emigrants; RTL, relative telomere length; RTx, renal transplantation; T=0, time of RTx; T=3, 3 months after RTx; T=6, 6 months after RTx; T_{NAIVE}, naive T cells.

in patients with a D+/R– configuration and the frequency of $CD8^+CD28^{null}$ T cells before transplant. We found that a higher frequency of $CD8^+CD28^{null}$ T cells is associated with a lower risk for the development of CMV viremia/disease. This might be related to the fact that these cells are preformed CMV-specific T cells in CMV-seronegative patients. A study by Lúcia et al. showed that CMV-specific interferon γ–producing memory T cells were present in CMV-seronegative patients and that high numbers of these cells provided clinical protection (123).

A study by Schürmann et al. showed that low counts of RTE and $CD4^+$ T cells before transplant were associated with the risk of PCP after RTx (124); however, after a multivariate regression analysis, only the pretransplant $CD4^+$ T lymphopenia and acute rejection within 3 months after RTx were associated with PCP (124). This study focused only on the development of PCP, whereas none of the patients in our study developed PCP. Furthermore, we analyzed each type of infection and did not concentrate on only one pathogen. Moreover, $CD4^+$ T lymphopenia was defined as a $CD4^+$ T-cell count of <300/µL (124). In our study, only 12 of 188 patients had such severe CD4+ T lymphopenia, and they were distributed equally over both groups. In addition, the study by Schürmann et al. included patients experiencing an acute rejection treated with T-cell depletion therapy, explaining the association found between acute rejection and the risk of PCP (124). As

expected, in this study, we also observed a strong association between T-cell depletion therapy and the risk of infections (14 of the 17 patients treated with T-cell depletion therapy developed an infection). Inclusion of these patients introduces a severe bias in the interpretation of the results; therefore, this group was excluded from our analyses.

Studies in the very old healthy population have identified an IRP as positively associated with increased morbidity and mortality (20, 112). This IRP was not predictive of an increased risk of infection within the first year after RTx in our cohort. The follow-up period for the very old healthy persons was much longer compared with our study. Furthermore, mortality as an end point covers all possible causes of death, not only infections. These differences could explain why an IRP is not a parameter for the prediction of infections in the first year after RTx.

Furthermore, we investigated whether the degree of premature T-cell ageing after transplant was associated with the risk of OIs and SIs. We did not find an association between the ageing parameters assessed at T=3 and infections thereafter; however, we observed that the infection group showed different posttransplant dynamics in T-cell subsets. We previously reported that the number of memory T cells decreases in the first months after RTx and thereafter is restored to pretransplant values between months 6 and 12 (125). The infection group showed a similar declining trend in memory T-cell numbers, but these returned much more to baseline (T=0) values at T=6, particularly within the $CD8^+$ compartment, compared with the group without infection. The different posttransplant T-cell dynamics were clearly caused by the infections. The significant rise in $CD8^+CD28^{null}$ T-cell percentage in the infection group, for example, was most likely caused by a primary CMV infection (27, 58, 126). A study by Calarota et al. showed that the drop in $CD4^+$ T cells coincided with the peak of OIs (119). Given our results, it is evident that a significant decrease in $CD4^+$ T cells occurs in all patients within this period, in which the majority of infections occur, and is not a causal association. In contrast, another study showed that a low $CD4^+$ T-cell count during the period after RTx, defined as a count <300/µL, predicted infection occurrence after RTx (127). Again, this observation might be influenced by inclusion of patients experiencing a rejection episode and treated with T-cell depletion therapy because low $CD4^+$ T-cell counts were associated with a more aggressive immunosuppressive regimen (127).

In contrast to the results of this study, other studies have reported that low values of RTE and RTL and a higher frequency of late-stage differentiated T cells are associated with infection development (113, 114); however, those patient cohorts were much more heterogeneous, and the use of anti–thymocyte globulin in these studies may have been an important confounder.

Another factor determining the course of T-cell ageing parameters is the intensity of the immunosuppressive regimen during the first 3 months. Most immunosuppression after RTx aims to attack the most aggressive T cells, namely, memory T cells (125, 128, 129). This probably explains the sharp decline in memory T-cell numbers in both groups during the first 3 months. Studies have shown that immunosuppressive drugs can

influence T-cell memory negatively because transplant recipients show lower or defective vaccination responses compared with healthy persons and show reactivation of latent viruses (128, 129).

In summary, various measures of T-cell ageing before and at 3 months after RTx are not predictive for infection risk after transplant. Memory T-cell dynamics after RTx are changed by infections but merely as a consequence of these infections, in particular, CMV infection.

A possible explanation for the lack of a relationship between T-cell ageing parameters and infections after transplant may lie in the fact that the relatively mild effects of T-cell ageing are overshadowed by the use of triple immunosuppression. Even in elderly patients with chronic diseases, true OIs are not observed, whereas a much younger patient who receives immunosuppressants is susceptible to these types of infections (116, 130). Another explanation may be found in the source of T cells that we used for our study. Tissue-derived resident T cells or those within secondary lymphoid organs may be a better representative population to gauge the immune function in ESRD patients than peripheral blood–derived T cells. The prematurely aged immune system in ESRD patients remains largely unchanged after successful RTx (125) and may become more relevant after a longer period in combination with immunosuppressive drugs. This could explain why a number of studies (127, 131, 132) have shown a relationship between persistent lymphopenia (not caused by T-cell depletion) after transplant and the long-term risk of cancer. An association with the consequences of infections >1 year after RTx will need further investigation.

In conclusion, this study analyzed the role of the prematurely aged T-cell system in ESRD patients before and after RTx in relation to the development of infections in a homogeneous patient population without T-cell depleting therapy. Our findings show that the tested T-cell parameters cannot be used to identify patients at risk for infection after transplantation.

DISCLOSURE

The authors declare no competing financial and commercial interests. This study was financially supported by the Dutch Kidney Foundation (KSPB.10.12).

SUPPLEMENTARY FILES

Supplementary Table 1. T-cell ageing parameters prior to renal transplantation (without CMV viremia/disease)

RT Patients (n=188)	No Infection (n=104) (55%)	Infection (n=59) (31%)	P
CD4 cells at T=0 (cells/μL)	688 (524 – 887)	714 (454 – 948)	0.871
CD4 naïve at T=0 (cells/μL)	207 (102 – 336)	151 (91 – 327)	0.622
CD4 memory at T=0 (cells/μL)	467 (357 – 573)	457 (297 – 648)	0.858
Naïve/memory CD4 at T=0	0.45 (0.23 – 0.70)	0.40 (0.19 – 0.69)	0.515
CD4 CM of CD4 memory at T=0 (%)	61.7 (49.8 – 73.5)	61.2 (52.0 – 70.5)	0.876
CD4 EM of CD4 memory at T=0 (%)	35.4 (24.5 – 45.8)	36.3 (28.1 – 46.6)	0.638
CD4 EMRA of CD4 memory at T=0 (%)	1.9 (0.7 – 4.2)	2.0 (1.1 – 4.0)	0.645
CD28null of CD4 at T=0 (%)	3.0 (0.5 – 8.9)	2.7 (0.3 – 10.9)	0.944
CD8 cells at T=0 (cells/μL)	390 (254 – 503)	342 (220 – 514)	0.281
CD8 naïve at T=0 (cells/μL)	64 (32 – 104)	45 (24 – 100)	0.162
CD8 memory at T=0 (cells/μL)	283 (189 – 405)	268 (151 – 361)	0.542
Naïve/memory CD8 at T=0	0.20 (0.10 – 0.53)	0.21 (0.09 – 0.53)	0.611
CD8 CM of CD8 memory at T=0 (%)	6.3 (3.5 – 13.5)	6.4 (2.7 – 12.1)	0.599
CD8 EM of CD8 memory at T=0 (%)	45.1 (29.8 – 58.5)	46.9 (26.0 – 59.1)	0.947
CD8 EMRA of CD8 memory at T=0 (%)	44.4 (27.8 – 64.9)	43.0 (26.3 – 64.0)	0.922
CD28null of CD8 at T=0 (%)	43.4 (23.8 – 61.2)	42.1 (20.5 – 60.1)	0.901
RTL CD4 at T=0	12.2 (9.8 – 14.7)	12.3 (8.9 – 15.3)	0.755
RTL CD8 at T=0	11.5 (9.5 – 14.6)	10.4 (8.6 – 15.8)	0.604
CD31 of naive CD4 at T=0 (%)	65.8 (55.8 – 75.4)	65.4 (52.9 – 71.8)	0.303
CD31 of naive CD8 at T=0 (%)	97.6 (94.5 – 98.8)	97.9 (94.0 – 99.1)	0.551

Data are presented as medians (interquartile range).

Supplementary Table 2. T-cell ageing parameters prior to renal transplantation for CMV D+/R+ & D-/R+ (moderate risk)

RT Patients (n=188)	No CMV viremia/disease (n=67) (36%)	CMV viremia/disease (n=8) (4%)	P
CD28null of CD4 at T=0 (%)	4.9 (2.6 – 11.4)	13.0 (4.5 – 19.7)	0.124
CD28null of CD8 at T=0 (%)	54.2 (36.8 – 66.9)	62.1 (46.5 – 67.2)	0.591
RTL CD4 at T=0	12.3 (9.6 – 15.4)	8.6 (6.4 – 13.7)	0.116
RTL CD8 at T=0	11.5 (9.3 – 14.4)	10.9 (8.2 – 15.8)	0.753
CD31 of naive CD4 at T=0 (%)	65.8 (56.6 – 76.3)	66.5 (47.0 – 82.7)	0.860
CD31 of naive CD8 at T=0 (%)	96.7 (93.9 – 98.7)	97.2 (96.2 – 99.2)	0.303

Data are presented as medians (interquartile range).

Supplementary Table 3. T-cell ageing parameters prior to renal transplantation for CMV D+/R- (high risk)

RT Patients (n=188)	No CMV viremia/disease (n=11) (6%)	CMV viremia/disease (n=16) (9%)	P
CD28null of CD4 at T=0 (%)	0.5 (0.2 – 4.8)	0.3 (0.2 – 2.0)	0.507
CD28null of CD8 at T=0 (%)	27.6 (11.2 – 37.9)	17.6 (10.4 – 21.7)	**0.036**
RTL CD4 at T=0	11.8 (10.2 – 18.2)	11.6 (9.8 – 16.8)	0.927
RTL CD8 at T=0	14.9 (11.8 – 16.3)	14.0 (10.1 – 17.4)	0.923
CD31 of naive CD4 at T=0 (%)	69.5 (48.2 – 72.1)	67.6 (45.8 – 76.9)	0.919
CD31 of naive CD8 at T=0 (%)	97.9 (94.3 – 99.4)	99.0 (91.8 – 99.4)	0.683

Data are presented as medians (interquartile range).

Supplementary Table 4. T-cell ageing parameters 3 months after renal transplantation (without CMV viremia/disease)

RT Patients (n=188)	No Infection (n=104) (55%)	Infection (n=26) (14%)	P
CD4 cells at T=3 (cells/μL)	511 (320 – 785)	410 (322 – 613)	0.187
CD4 naïve at T=3 (cells/μL)	182 (91 – 328)	146 (94 – 300)	0.574
CD4 memory at T=3 (cells/μL)	312 (215 – 485)	251 (162 – 363)	0.078
Naïve/memory CD4 at T=3	0.59 (0.30 – 0.92)	0.73 (0.27 – 1.02)	0.490
CD4 CM of CD4 memory at T=3 (%)	63.6 (50.7 – 73.0)	64.1 (57.1 – 73.4)	0.974
CD4 EM of CD4 memory at T=3 (%)	32.7 (24.0 – 42.9)	33.8 (23.6 – 40.5)	0.947
CD4 EMRA of CD4 memory at T=3 (%)	2.4 (1.1 – 4.7)	2.6 (1.4 – 3.4)	0.993
CD28null of CD4 at T=3 (%)	3.0 (1.1 – 7.6)	3.3 (0.6 – 10.2)	0.725
CD8 cells at T=3 (cells/μL)	272 (180 – 441)	188 (122 – 364)	**0.031**
CD8 naïve at T=3 (cells/μL)	64 (27 – 126)	48 (18 – 74)	0.053
CD8 memory at T=3 (cells/μL)	188 (116 – 328)	121 (70 – 293)	0.098
Naïve/memory CD8 at T=3	0.31 (0.14 – 0.81)	0.29 (0.07 – 0.68)	0.472
CD8 CM of CD8 memory at T=3 (%)	7.4 (3.7 – 12.3)	6.4 (3.3 – 12.8)	0.753
CD8 EM of CD8 memory at T=3 (%)	42.9 (28.8 – 54.5)	48.2 (31.5 – 57.4)	0.282
CD8 EMRA of CD8 memory at T=3 (%)	49.6 (29.7 – 65.2)	41.8 (29.6 – 55.1)	0.344
CD28null of CD8 at T=3 (%)	39.2 (20.4 – 59.9)	35.5 (14.9 – 64.1)	0.837
RTL CD4 at T=3	13.3 (11.5 – 16.9)	11.7 (8.5 – 16.6)	0.163
RTL CD8 at T=3	13.0 (10.2 – 16.1)	12.5 (9.3 – 16.4)	0.520
CD31 of naive CD4 at T=3 (%)	65.0 (51.2 – 75.7)	65.6 (49.6 – 71.8)	0.629
CD31 of naive CD8 at T=3 (%)	98.0 (95.5 – 99.1)	97.7 (93.8 – 99.3)	0.919

Data are presented as medians (interquartile range).

Supplementary Table 5. Correlation between T-cell parameters and infection after 3 months (without CMV viremia/disease)

T-cell parameter	ρ	P
CD4 memory at T=3 (cells/μL)	-0.07	0.409
CD8 cells at T=3 (cells/μL)	-0.11	0.235
CD8 naïve at T=3 (cells/μL)	-0.16	0.063

Chapter 5

LYMPH NODE AND CIRCULATING T-CELL CHARACTERISTICS ARE STRONGLY CORRELATED IN END-STAGE RENAL DISEASE PATIENTS, BUT HIGHLY DIFFERENTIATED T CELLS RESIDE WITHIN THE CIRCULATION

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ABSTRACT

Background

Ageing is associated with changes in the peripheral T-cell immune system, which can be significantly influenced by latent cytomegalovirus (CMV) infection. To what extent changes in circulating T-cell populations correlate with T-cell composition of the lymph node (LN) is unclear, but is crucial for a comprehensive understanding of the T-cell system.

Methods

T cells from peripheral blood (PB) and LN of end-stage renal disease patients were analyzed for frequency of recent thymic emigrants using CD31 expression and T-cell receptor excision circle content, relative telomere length and expression of differentiation markers.

Results

Compared with PB, LN contained relatively more $CD4^+$ than $CD8^+$ T cells ($p<0.001$). The percentage of naïve and central memory $CD4^+$ and $CD8^+$ T cells and thymic output parameters, showed a strong linear correlation between PB and LN. Highly differentiated $CD28^{null}$ T cells, being $CD27^-$, $CD57^+$ or programmed death 1 ($PD\text{-}1^+$), were found almost exclusively in the circulation but not in LN. An age-related decline in naïve $CD4^+$ and $CD8^+$ T-cell frequency was observed ($p=0.035$ and $p=0.002$ respectively) within LN, concomitant with an increase in central memory $CD8^+$ T cells ($p=0.033$). Latent CMV infection increased dramatically the frequency of circulating terminally differentiated T cells, but did not alter T-cell composition and ageing parameters of LN significantly.

Conclusion

Overall T-cell composition and measures of thymic function in PB and LN are correlated strongly. However, highly differentiated $CD28^{null}$ T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are found almost exclusively within the circulation.

INTRODUCTION

One of the dominant aspects of the ageing process in the T-cell immune system, is a decreased thymic output of newly formed T cells, named recent thymic emigrants (RTE). This is evident from a decrease in circulating naive T cells expressing CD31, and by a decrease in T-cell receptor excision circles (TREC) content (16-18, 133, 134). Furthermore, an increase in age leads to a decline in the frequency of circulating naive T cells, while the memory T-cell compartment may contain more terminally differentiated T cells (20, 21). This progressive T-cell differentiation is also marked by the loss of the co-stimulatory molecule CD28 on the surface of memory T cells (27), which enables these cells to become activated without the need for co-stimulation. Next to the loss of CD28, the loss of CD27, and the expression of CD57 and PD-1 are indicators of terminal T-cell differentiation (24-26). Lastly, the increased number of rounds of T-cell proliferation and differentiation reduces the telomere length of T cells (45, 46).

Cytomegalovirus (CMV) infection needs to be taken into account when the T-cell compartment is analyzed, as this infection may cause a significant imprint on the differentiation status of circulating T cells (27, 42). In general, it leads to an expansion of late-differentiated $CD4^+$ and $CD8^+$ T cells and an inverted CD4:CD8 T-cell ratio (43). Furthermore, CMV infection may lead to a reduction in telomere length of T cells (135).

The impact of an aged circulating T-cell population has been studied, for example, in relation to the risk for infection, decreased vaccination response and cardiovascular events (29, 33, 136). In addition, the presence of highly differentiated $CD28^{null}$ T cells in the peripheral blood has gained considerable interest in the field of solid organ transplantation as they may be instrumental in co-stimulatory blockade resistant rejection and long-term allograft (dys)function (29, 31, 34, 63, 137). Analysis of the T-cell population in lymph nodes (LNs) might prove to be of additional/higher value, as immune responses are initiated in secondary lymphoid organs. Until now, little is known about how changes in circulatory T cells relate to changes in the LN, and only a few studies have investigated the effect of ageing in secondary lymphoid organs (49, 50).

In this study, we investigated the relation between T-cell parameters of the LN and PB compartments in end-stage renal disease (ESRD) patients. More specifically, ageing parameters such as thymic output, relative telomere length (RTL), terminal differentiation markers and the influence of CMV infection on these were studied. Our results show that overall T-cell composition and measures of thymic function are strongly correlated between PB and LN. However highly differentiated $CD28^{null}$ T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are virtually absent in LNs.

MATERIALS AND METHODS

Study population

Patients prior to kidney transplantation in the period from August 2015 to March 2016 were eligible for participation. All patients gave written informed consent to participate in this study. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2015-301) and was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Clinical variables were assessed as shown in Table 1, including age, gender, CMV-seropositivity, underlying cause of renal failure, dialysis prior to transplantation and dialysis vintage (defined as time on dialysis since the start of any type of dialysis).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by using Ficoll-Paque Plus (GE healthcare, Uppsala, Sweden). Blood was obtained from renal transplant recipients one day prior to RT. The isolated PBMCs were stored at -150°C with a minimum amount of 10×10^6 cells per vial. The median frequency of lymphocytes within heparinized blood amounted to 21.9% (17.7%–26.6%) and following density gradient centrifugation this frequency amounted to 72.6% (64.7%–76.7%).

LNMC isolation

Iliac LNs were isolated from tissue which was obtained as waste material from renal transplant recipients at the time of implantation of the renal allograft. Upon arrival of the material at the laboratory, excessive fat tissue around the LNs was removed. Next, the LNs were minced and subsequently mashed through a 70 µm sieve to obtain a cell suspension. Then, the cell suspension was centrifuged several times to remove platelets and remaining fat tissue. The isolated lymph node mononuclear cells (LNMCs) were stored at -150°C with a minimum amount of 10×10^6 cells per vial. The median yield of a lymph node was 20×10^6 cells. The median frequency of lymphocytes within LNMNCs amounted 94.8% (92.0% - 96.3%).

T-cell differentiation status

T-cell differentiation status was determined through a whole blood staining and on freshly isolated LNMCs, which was based upon the study by Sallusto et al. (22) as described in detail previously (11). These cell samples were measured on the fluorescence activated cell sorter (FACS)Canto II (BD Biosciences, San Jose, CA, USA) and analyzed with the FACS Diva software version 6.1.2 (BD Biosciences). Briefly, differential cell surface expression of CD45RO (pan-T cell marker for memory T cells) and CCR7 (a chemokine receptor involved in homing of T cells to various secondary lymphoid organs such as lymph nodes) was used to distinguish naive from different memory T-cell populations. Memory T cells differentiate into central-memory T cells ($CD45RO^+CCR7^+$ T cells, able to access the lymphoid tissue), effector-memory T cells ($CD45RO^+CCR7^-$ T cells) and EMRA T cells (highly differentiated T cells re-expressing CD45RA but $CCR7^-$) (22). A typical example of the gating strategy, is shown in supplementary Figure 1.

In addition to this definition of memory T cells, the expression of CD28, CD27, CD57 and PD-1 was used to divide the T-cell compartment in early or late differentiated T cells. A typical example of the gating strategy, is shown in supplementary Figure 2. Essentially, the naive and central-memory T cells are early differentiated T cells and still express the costimulatory molecules CD28 and CD27. EM and EMRA T cells may progressively lose CD28 and subsequently CD27 expression and gain expression of CD57 and PD-1 (24-26, 138). Next to this, examining expression of CD28 together with CD27 is of additional value, as the order of loss of these molecules differs between $CD4^+$ and $CD8^+$ T cells (i.e. $CD4^+$ T cells first lose CD27 before losing CD28, while for the $CD8^+$ T cells this is the other way around) (75, 139, 140). Early differentiated T cells were defined as $CD28^+CD27^+$, $CD28^+PD\text{-}1^-$ and $CD28^+CD57^-$ T cells. T cells that were $CD28^{null}CD27^-$, $CD28^{null}PD\text{-}1^+$ and $CD28^{null}CD57^+$ were defined as late differentiated memory T cells. This measurement was performed on the Navios flow cytometer (Beckman Coulter) and the obtained data were analyzed with the Kaluza software version 1.3 (Beckman Coulter). The T cells were analyzed with DuraClone IM T-cell subsets tubes (Beckman Coulter), which were coated with allophycocyanin Alexa Fluor 750 (APC-A750) anti-CD3, allophycocyanin (APC) labeled anti-CD4, Alexa Fluor 700 (A700) labeled anti-CD8, fluorescein isothiocyanate (FITC) labeled anti-CD45RA, phycoerythrin (PE) labeled anti-CCR7, phycoerythrin Texas red (ECD) labeled anti-CD28, phycoerythrin-cyanine 7 (PE-Cy7) labeled anti-CD27, phycoerythrin-cyanine 5.5 (PE-Cy5.5) labeled anti-PD1, pacific blue (PacB) labeled anti-CD57 and krome orange (KO) labeled anti-CD45.

Relative Telomere Length

To determine the RTL of $CD4^+$ and $CD8^+$ T cells, flow fluorescent in situ hybridization was performed on thawed PBMCs and LNMCs as described in detail previously (11).

Assessment of recent thymic emigrants using CD31 and TREC content

$CD31^+$ naive T cells were assessed by flow cytometry as a measure of recent thymic emigrants (RTE), as described previously (125). TREC content was determined using 1×10^6 snap-frozen PBMCs and LNMCs. DNA was isolated from these snap-frozen samples and the TREC content, depicted by DCT (which is inversely related to the TREC content), was determined using quantitative polymerase chain reaction (PCR) as described previously (13).

Statistical analysis

All variables are presented as medians with interquartile ranges. The differences between paired samples (i.e. PB and LN T-cell ageing parameters of same ESRD patients) were

analyzed using the Wilcoxon signed-rank test. Differences between continuous variables from two independent groups (i.e. CMV-seropositive versus CMV-seronegative ESRD patients) were assessed with the Mann–Whitney U test. Correlations between PB and LN were calculated using the Spearman's rank correlation. Relationships between age and the different T-cell ageing parameters were assessed using a linear regression analysis. The significance level (p-value) was two-tailed and an α of 0.05 was used for all analyses. Statistical analyses were performed using SPSS® version 21.0 for Windows® (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (San Diego, CA, USA). Figures were created with GraphPad Prism 5.

RESULTS

Patient characteristics

Patient characteristics (*n*=38) are shown in Table 1. The median patient age was 58 years. Most of the patients were CMV immunoglobulin (Ig)G+ (74%). The major cause of ESRD was nephrosclerosis/atherosclerosis/hypertension (29%), followed by polycystic kidney disease (21%), which together accounted for half of the cases.

CD4⁺ T-cell composition of the lymph node and peripheral blood

The median frequency of CD4⁺ T cells was significantly higher in LN samples compared with the PB (p<0.001) (Figure 1A), but the relative distribution of CD4⁺ naive T cells was similar (Figure 1B). However, compared with PB, the LN contained higher frequencies of CD4⁺ CM T cells (p<0.001) (Figure 1C) and lower frequencies of CD4⁺ EM T cells and

Table 1. Patient characteristics

	RT Patients (*n*=38)
Age	58 (45 – 65)
Male	24 (63%)
CMV-seropositivity	28 (74%)
Underlying disease	
Nephrosclerosis/atherosclerosis/hypertension	11 (29%)
Primary glomerulopathies	5 (13%)
Diabetes	7 (18%)
Urinary tract infections/stones	0 (0%)
Reflux nephropathy	0 (0%)
Polycystic Kidney Disease	8 (21%)
Other	3 (8%)
Unknown	4 (11%)
Dialysis prior to transplantation	24 (63%)

Data are presented as median (interquartile range) or as number (percentage). RT indicates renal transplantation; and CMV, cytomegalovirus.

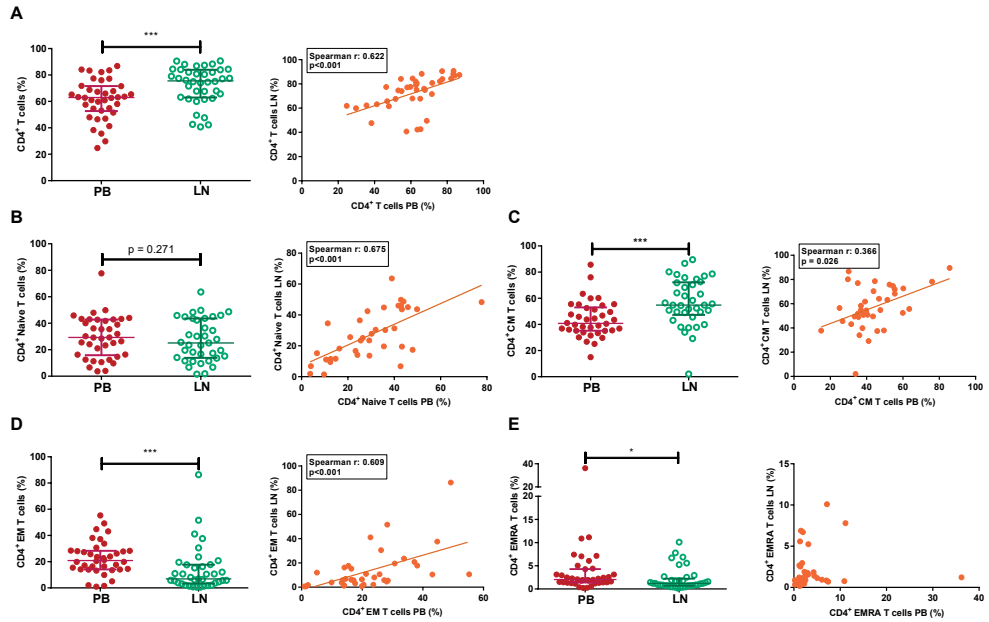


Figure 1. CD4⁺ T-cell subsets in the peripheral blood (PB) and the lymph node (LN) and the correlation between PB and LN. The frequencies and correlations of (A) CD4⁺ T cells within CD3⁺ T cells, (B) CD4⁺ naive T cells within CD4⁺ T cells, (C) CD4⁺ central memory (CM) T cells within CD4⁺ T cells, (D) CD4⁺ effector memory (EM) T cells within CD4⁺ T cells and (E) CD4⁺ effector memory CD45RA⁺ (EMRA) T cells within CD4⁺ T cells are represented. Frequencies of cells are depicted as individual percentages as well as median and interquartile range (left plots). A Spearman's rho was shown only when a significant correlation was present; *n*=38 for peripheral blood and *n*=38/37 for lymph node samples. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

effector memory CD45RA⁺ (EMRA) T cells (p<0.001 and p=0.026 respectively) (Figures 1D-E). Except for the frequency of CD4⁺ EMRA T cells, frequencies of CD4⁺ T cells, CD4⁺ naive T cells, CD4⁺ CM T cells and CD4⁺ EM T cells showed a remarkable good correlation between PB and LN (Figure 1).

Next, the early versus late T-cell differentiation was analyzed by measuring the expression of CD27, PD-1 and CD57 on CD28⁺ and CD28^{null} T cells (Table 2). Within the CD4⁺ T-cell population, the frequency of CD28^{null} T cells was significantly lower in LN compared with PB (1.0%, range 0.6%-1.6% vs 1.8%, range 0.7-8.8; p=0.001) and late memory T cells subpopulations, either defined as CD28^{null}CD27⁻ T cells, CD28^{null}PD-1⁺ T cells or CD28^{null}CD57⁺ T cells, were virtually absent in LN (p<0.001 for all comparisons). Instead, the percentage of CD4⁺ T cells with full expression of CD28 and CD27 (CD28⁺CD27⁺) was significantly higher in the LN (Table 2).

In summary, the composition of the CD4⁺ T-cell compartment in PB and LN are highly interrelated, except for the absence of late differentiated T cells, which almost exclusively appear in PB.

Table 2. CD27, PD-1 and CD57 expression on CD28^{null} T cells in the peripheral blood and lymph node

	PB (n=38)	LN (n=33)	p
CD4$^+$ T cells			
CD28$^+$CD27$^+$ (%)	87.4 (81.5 – 94.3)	93.8 (91.6 – 96.1)	<0.001
CD28^{null}CD27$^-$ (%)	1.1 (0.2 – 7.5)	0.3 (0.1 – 0.6)	<0.001
CD28$^+$PD–1$^-$ (%)	78.5 (71.0 – 84.0)	74.2 (65.8 – 81.6)	0.077
CD28^{null}PD–1$^+$ (%)	1.0 (0.3 – 7.3)	0.4 (0.3 – 0.9)	0.004
CD28$^+$CD57$^-$ (%)	96.7 (90.1 – 98.7)	97.4 (96.0 – 98.2)	0.063
CD28^{null}CD57$^+$ (%)	0.8 (0.1 – 5.9)	0.1 (0.0 – 0.2)	<0.001
CD8$^+$ T cells			
CD28$^+$CD27$^+$ (%)	54.8 (36.7 – 73.7)	89.8 (84.1 – 92.6)	<0.001
CD28^{null}CD27$^-$ (%)	28.7 (10.8 – 49.3)	1.9 (1.0 – 2.6)	<0.001
CD28$^+$PD–1$^-$ (%)	43.1 (25.9 – 57.5)	63.2 (49.5 – 75.1)	<0.001
CD28^{null}PD–1$^+$ (%)	12.4 (6.9 – 18.4)	2.4 (1.6 – 5.1)	<0.001
CD28$^+$CD57$^-$ (%)	56.2 (37.5 – 73.9)	87.3 (80.2 – 89.7)	<0.001
CD28^{null}CD57$^+$ (%)	26.6 (13.4 – 39.1)	1.5 (1.0 – 2.3)	<0.001

Data are presented as medians (interquartile range). PB indicates peripheral blood; and LN, lymph node.

CD8$^+$ T-cell composition of the lymph node and peripheral blood

The frequency of CD8$^+$ T cells was significantly lower in LN than in PB with a relative higher proportion of naive T cells (p<0.001 for both variables) (Figure 2A-B). The CD8$^+$ memory T-cell population in LN contained relatively more CM T cells (p<0.001) (Figure 2C) and less highly differentiated CD8$^+$ EMRA T cells (p<0.001) (Figure 2E).

All CD8$^+$ T cell subsets, except for EM T cells, were positively correlated between the two different compartments (Figure 2). Specifically, the frequency of total CD8$^+$ T cells, CD8$^+$ naive T cells and CD8$^+$ CM T cells showed a very close correlation.

Differentiated CD8$^+$CD28^{null} T cells were significantly less frequent in LN than in PB (10.6%, range 7.5%–15.0% vs 41.5%, range 23.8%–64.0%; p<0.001).

The expression of CD27, PD-1 and CD57 in relation to CD28 expression showed similar results within the CD8$^+$ T-cell population as obtained for the CD4$^+$ T cells (Table 2). The frequencies of late differentiated T cells (CD8$^+$CD28^{null} T cells lacking CD27, expressing PD-1 or CD57) were high in PB, but few of these cells were found in LN (p<0.001). In contrast, LN contained significantly more T cells expressing CD28 and CD27.

In summary, similar to the CD4$^+$ T cells, the composition of the CD8$^+$ T-cell compartment of PB and LN was highly interrelated, but late differentiated CD8$^+$CD28^{null} T cells were confined to the circulating CD8$^+$ T-cell pool.

Recent thymic emigrants and RTL in the lymph node and peripheral blood

Frequencies of CD31$^+$ naive T cells were significantly lower in LN than in PB for both CD4$^+$ and CD8$^+$ T cells (p<0.001 and p=0.008, respectively) (Figure 3A and B). Frequencies of

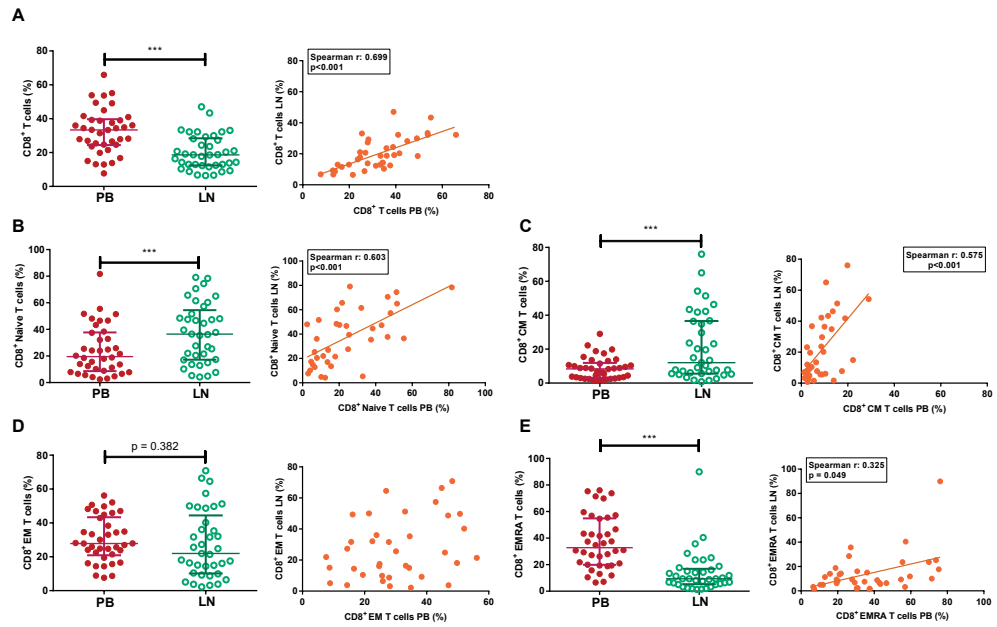


Figure 2. CD8$^+$ T-cell subsets in the peripheral blood (PB) and the lymph node (LN) and the correlation between PB and LN. The frequencies and correlations of (A) CD8$^+$ T cells within CD3$^+$ T cells, (B) CD8$^+$ naive T cells within CD8$^+$ T cells, (C) CD8$^+$ central memory (CM) T cells within CD8$^+$ T cells, (D) CD8$^+$ effector memory (EM) T cells within CD8$^+$ T cells and (E) CD8$^+$ effector memory CD45RA$^+$ (EMRA) T cells within CD8$^+$ T cells are represented. Frequencies of cells are depicted as individual percentages as well as median and interquartile range (left plots). A Spearman's rho was shown only when a significant correlation was present; *n*=38 for peripheral blood and *n*=38/37 for lymph node samples. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

CD31$^+$ CD4$^+$ and CD31$^+$ CD8$^+$ naive T cells in PB and LN were highly correlated (p<0.001 for both variables) (Figure 3A and B). Furthermore, the TREC content in the lymphocyte population, which is another measure of the number of RTE, was significantly higher (i.e. lower ΔCT) in the LN (p=0.002) (Figure 3C) compared with PB. The TREC content also showed a significant positive correlation between PB and LN (Figure 3C). Combining both parameters, a significant negative correlation between the frequency of CD31$^+$ CD4$^+$ and CD8$^+$ naive T cells and ΔCT in both the PB and LN was observed (Supplementary Figure 3). Interestingly, the RTL of both CD4$^+$ as well as CD8$^+$ T cells was similar between PB and LN (Figure 3D and E).

Age-related changes of T-cell characteristics in the lymph node

Figure 4 shows the T-cell characteristics that were significantly associated with age. A negative association between age and frequency of CD4$^+$ naive T cells was observed (B= –0.42, p=0.035) (Figure 4A), while the frequencies of CD4$^+$ memory T cells increased

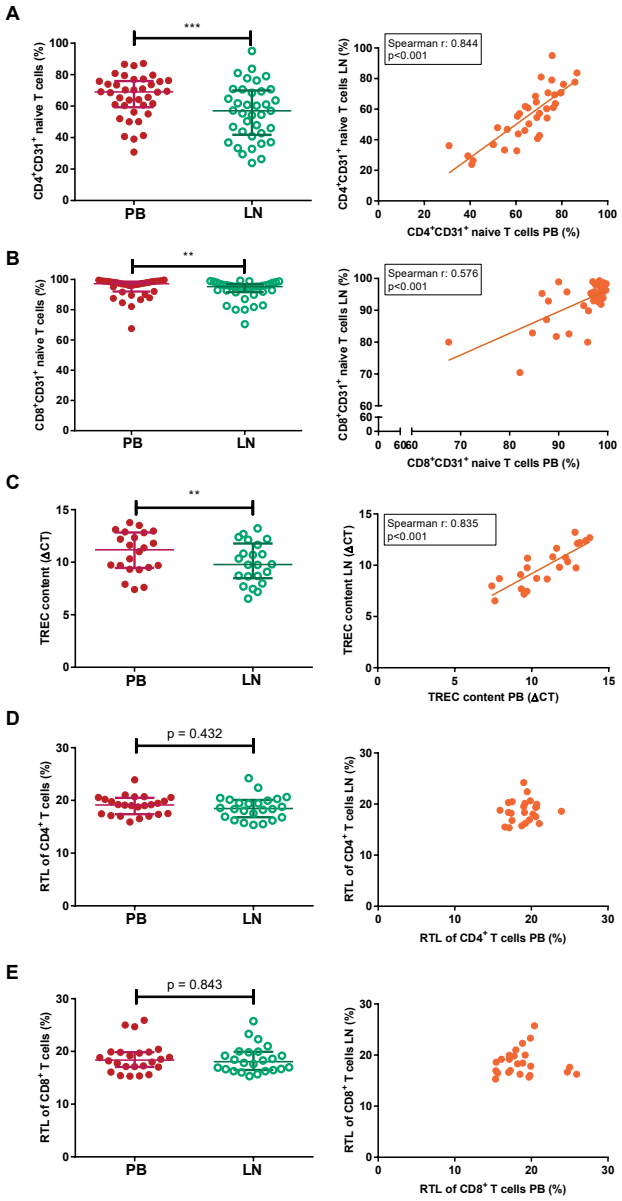


Figure 3. CD31⁺ naive T cell frequency, T cell receptor excision circles (TREC) content and relative telomere length (RTL) in the peripheral blood (PB) and lymph node (LN) with correlations. The frequencies and correlations of (A) CD4⁺CD31⁺ naive T cells, (B) CD8⁺CD31⁺ naive T cells, (C) TREC content, (D) CD4⁺ RTL and (E) CD8⁺ RTL for both the peripheral blood and the lymph node are represented. Frequencies of CD4⁺ and CD8⁺CD31⁺ naive T cells and RTL are depicted as percentages as well as median and interquartile range. The ΔCT is related inversely to the TREC content; *n*=38 for peripheral blood and *n*=37 for lymph node samples with regard to the analysis of CD4⁺CD31⁺ naive T cells and CD8⁺CD31⁺ naive T cells; *n*=22 for analysis of the TREC content and *n*=24 for the analysis of RTL in both T cell subsets.. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

with age (B=0.42, p=0.035) (Figure 4B). The expression of CD27, PD-1 and CD57 in combination with CD28 expression within the CD4⁺ T-cell population did not show any associations with age (data not shown).

Similarly, frequencies of naive CD8⁺ T cells were negatively associated with age (B= –0.82, p=0.002) (Figure 4C), whereas those of CD8⁺ memory T cells revealed a positive association with age (B=0.59, p=0.023) (Figure 4D). Next to this, also the frequency of CD8⁺ CM T cells was positively associated with age (B=0.40, p=0.033) (Figure 4E). Within the CD8⁺ T-cell population, frequencies of early differentiated T cells, i.e. CD28⁺CD27⁺ and CD28⁺PD1⁻ T cells were decreased with increasing age (B= –0.20, p=0.028 and B= –0.50, p=0.043 respectively).

Assessment of thymic output also revealed age-associated changes within the LN. Frequencies of CD4⁺ RTE decreased with age (B= –0.62, p=0.004) (Figure 4F) as did the TREC content (i.e. higher ΔCT) (B=0.09, p=0.003) (Figure 4G). Conversely, the RTL of both the CD4⁺ and the CD8⁺ T cells in the LN showed no association with age (data not shown).

CMV infection does not change lymph node T-cell characteristics

In accordance with previous studies (27, 42, 43), CMV infection altered the peripheral T-cell compartment leading to relatively more CD8⁺ T cells, as well as increased frequencies of CD4⁺CD28^{null} and CD8⁺CD28^{null} T cells showing decreased expression of CD27 and increased expression of PD-1 and CD57 (Table 3 & Table 4).

However, CMV infection did not affect the composition of CD4⁺ (Table 3) and CD8⁺ T cells (Table 4) within LN.

In addition, no association was seen between CMV infection and thymic output parameters in LN, with the frequencies of CD4⁺CD31⁺ and CD8⁺CD31⁺ naive T cells and the TREC content being similar between CMV+ and CMV– patients (data not shown). Similarly, no effect of CMV on RTL of CD4⁺ and CD8⁺ T cells within LN was observed (data not shown).

In summary, despite a major effect of CMV infection on peripheral blood T-cell composition, this was not paralleled by similar effects within LN.

DISCUSSION

In this study we investigated, whether the ageing parameters of the circulating T-cell population, correlates with the T-cell characteristics of LN and the effect of CMV on PB and LN composition. Our main conclusions are that the T-cell composition and measures of thymic function in PB and LN are interrelated, but that the highly differentiated CD28^{null} T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are almost exclusively found within the circulation. This study is the first in which a pairwise and a more in depth comparison was performed between PB and LN for different T-cell ageing parameters in ESRD patients. Next to the different T-cell subsets,

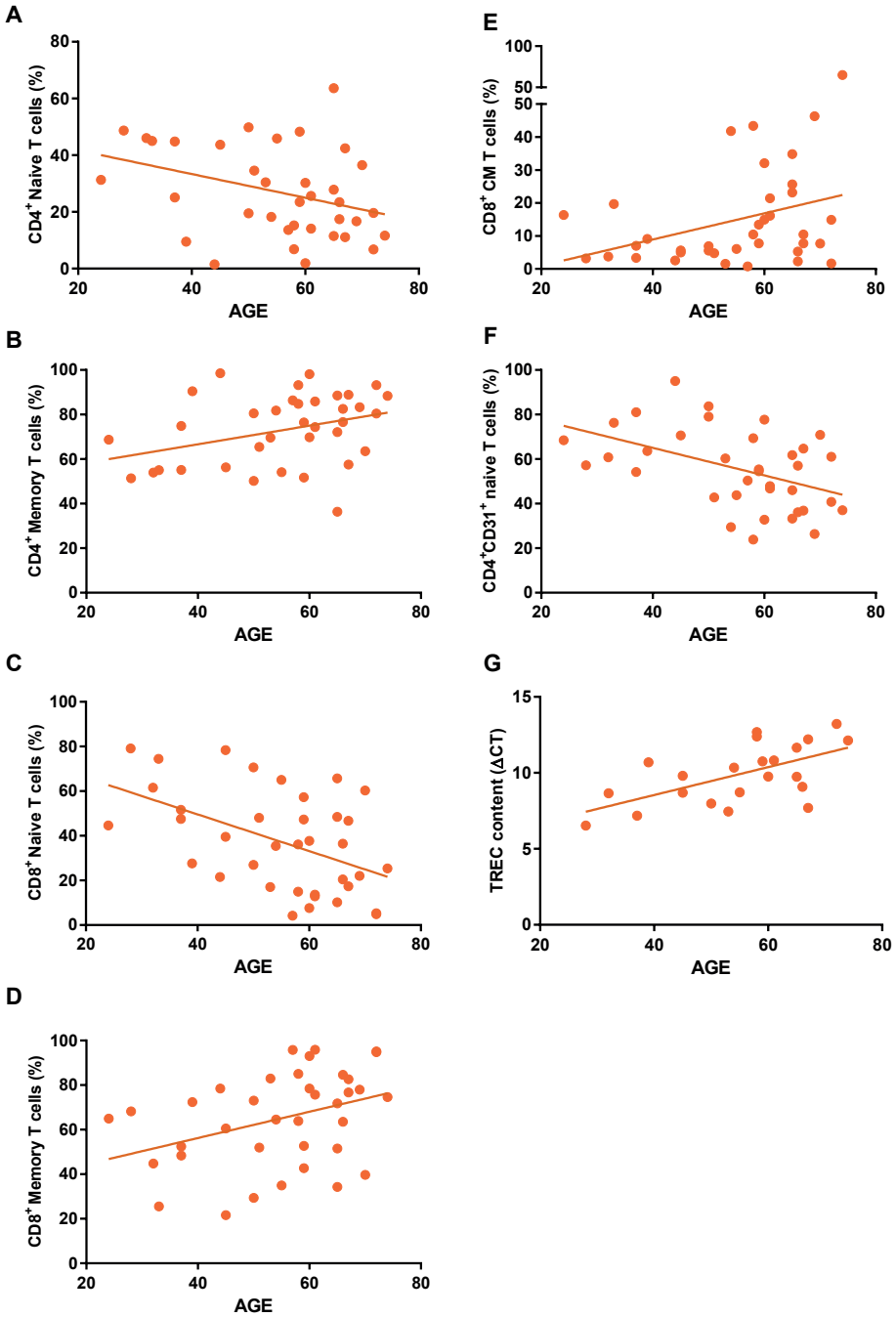


Figure 4. Effect of age on T-cell ageing parameters in the lymph node (LN). Linear regression analysis between age and (A) CD4⁺ naive T cells, (B) CD4⁺ memory T cells, (C) CD8⁺ naive T cells, (D) CD8⁺ memory T cells, (E) CD8⁺ central memory (CM) T cells and (F) CD4⁺CD31⁺ naive T cells and (G) T-cell receptor excision circles (TREC) content are represented. Frequencies of cells are depicted with percentages and the TREC content is depicted with ΔCT. Only significant associations are shown.

Table 3. CD4⁺ T-cell differentiation status in lymph node and peripheral blood for CMV+ and CMV– patients

	n	CMV+	n	CMV–	p
Lymph node					
CD4⁺ T cells (%)	28	74.3 (62.5 – 80.1)	10	83.0 (68.6 – 87.0)	0.094
CD4⁺ naive T cells (%)	27	30.2 (13.6 – 43.7)	10	18.9 (12.3 – 30.7)	0.313
CD4⁺ memory T cells (%)	27	69.8 (56.3 – 86.4)	10	81.1 (69.3 – 87.7)	0.313
CD4⁺ CM T cells (%)	27	54.8 (43.1 – 68.3)	10	63.8 (49.5 – 76.5)	0.383
CD4⁺ EM T cells (%)	27	10.5 (4.6 – 17.8)	10	4.7 (1.9 – 22.4)	0.494
CD4⁺ EMRA T cells (%)	27	1.1 (0.8 – 1.9)	10	1.8 (0.6 – 2.6)	0.620
CD4⁺CD28^{null} T cells (%)	27	1.1 (0.7 – 1.8)	10	0.7 (0.3 – 1.1)	0.078
CD4⁺CD28⁺CD27⁺ (%)	23	93.9 (92.2 – 97.3)	10	92.4 (89.9 – 96.0)	0.248
CD4⁺CD28^{null}CD27⁻ (%)	23	0.3 (0.1 – 0.6)	10	0.4 (0.1 – 0.5)	0.739
CD4⁺CD28⁺PD–1⁻ (%)	23	73.9 (66.8 – 78.2)	10	79.7 (64.4 – 84.6)	0.281
CD4⁺CD28^{null}PD–1⁺ (%)	23	0.4 (0.3 – 1.0)	10	0.6 (0.3 – 0.9)	0.681
CD4⁺CD28⁺CD57⁻ (%)	23	97.1 (96.6 – 98.3)	10	97.6 (93.4 – 98.2)	0.652
CD4⁺CD28^{null}CD57⁺ (%)	23	0.1 (0.0 – 0.2)	10	0.0 (0.0 – 0.2)	0.246
Peripheral blood					
CD4⁺ T cells (%)	28	60.3 (48.8 – 64.8)	10	76.7 (68.5 – 83.7)	**<0.001**
CD4⁺ naive T cells (%)	28	28.9 (13.1 – 42.2)	10	32.4 (19.9 – 42.8)	0.703
CD4⁺ memory T cells (%)	28	71.1 (57.8 – 86.9)	10	67.6 (57.2 – 80.2)	0.703
CD4⁺ CM T cells (%)	28	39.5 (35.3 – 50.9)	10	44.0 (33.8 – 54.8)	0.655
CD4⁺ EM T cells (%)	28	22.5 (15.7 – 30.1)	10	17.3 (13.8 – 25.1)	0.380
CD4⁺ EMRA T cells (%)	28	2.0 (1.3 – 4.9)	10	2.1 (1.4 – 3.3)	0.934
CD4⁺CD28^{null} T cells (%)	28	5.6 (1.3 – 11.5)	10	0.6 (0.3 – 0.8)	**<0.001**
CD4⁺CD28⁺CD27⁺ (%)	28	83.9 (77.8 – 92.7)	10	94.2 (89.7 – 9.5)	**0.012**
CD4⁺CD28^{null}CD27⁻ (%)	28	3.9 (0.7 – 9.2)	10	0.1 (0.0 – 0.2)	**<0.001**
CD4⁺CD28⁺PD–1⁻ (%)	28	77.7 (71.0 – 82.8)	10	81.4 (71.1 – 89.0)	0.214
CD4⁺CD28^{null}PD–1⁺ (%)	28	2.3 (0.6 – 9.0)	10	0.1 (0.0 – 0.3)	**<0.001**
CD4⁺CD28⁺CD57⁻ (%)	28	93.9 (88.7 – 97.4)	10	98.9 (97.8 – 99.4)	**<0.001**
CD4⁺CD28^{null}CD57⁺ (%)	28	2.7 (0.6 – 6.5)	10	0.0 (0.0 – 0.2)	**<0.001**

Data are presented as medians (interquartile range). CMV indicates cytomegalovirus; CM, central memory, EM, effector memory; and EMRA, effector memory CD45RA⁺.

we included analyses of other differentiation markers such as CD27, PD-1 and CD57 in combination with CD28. Furthermore, we also analyzed the TREC content and RTL of the T cells in both the lymph node and the peripheral blood.

In previous studies it was shown that the PB compartment of ESRD patients displayed distinct changes that resemble the T-cell ageing process in elderly healthy individuals. It is associated with loss of naive T cells, an inverted CD4:CD8 ratio and an increase in highly differentiated T cells, lacking the co-stimulatory molecule CD28 (11, 13, 30, 56, 141). The T-cell composition of LNs of the same patients, showed a higher frequency of CD4⁺ T cells and a lower frequency of CD8⁺ T cells compared with PB. The high frequency of

Table 4. CD8⁺ T-cell differentiation status in lymph node and peripheral blood for CMV+ and CMV– patients

	n	CMV+	n	CMV–	p
Lymph node					
CD8⁺ T cells (%)	28	19.0 (13.2 – 29.5)	10	13.0 (8.7 – 22.9)	0.159
CD8⁺ naive T cells (%)	27	39.5 (20.5 – 51.6)	10	28.5 (11.0 – 58.4)	0.281
CD8⁺ memory T cells (%)	27	60.5 (48.4 – 79.6)	10	71.5 (41.6 – 89.1)	0.281
CD8⁺ CM T cells (%)	27	12.0 (5.6 – 36.5)	10	11.3 (4.6 – 44.2)	0.959
CD8⁺ EM T cells (%)	27	21.9 (9.9 – 36.1)	10	26.5 (14.3 – 53.7)	0.442
CD8⁺ EMRA T cells (%)	27	8.8 (5.0 – 17.7)	10	12.7 (7.4 – 19.2)	0.330
CD8⁺CD28^{null} T cells (%)	27	10.7 (7.4 – 16.7)	10	9.1 (6.8 – 13.4)	0.365
CD8⁺CD28⁺CD27⁺ (%)	23	90.9 (83.3 – 92.7)	10	86.5 (84.1 – 91.4)	0.357
CD8⁺CD28^{null}CD27⁻ (%)	23	1.9 (1.2 – 3.4)	10	1.4 (0.8 – 2.5)	0.240
CD8⁺CD28⁺PD–1⁻ (%)	23	63.2 (50.2 – 74.8)	10	63.7 (46.4 – 76.0)	0.953
CD8⁺CD28^{null}PD–1⁺ (%)	23	2.2 (1.5 – 5.4)	10	3.5 (2.1 – 5.3)	0.240
CD8⁺CD28⁺CD57⁻ (%)	23	87.3 (81.6 – 90.1)	10	87.4 (73.6 – 89.3)	0.681
CD8⁺CD28^{null}CD57⁺ (%)	23	1.6 (1.1 – 2.7)	10	1.3 (1.0 – 1.8)	0.248
Peripheral blood					
CD8⁺ T cells (%)	28	35.5 (27.9 – 41.4)	10	18.3 (13.1 – 27.3)	**0.002**
CD8⁺ naive T cells (%)	27	18.7 (7.7 – 25.8)	10	32.5 (14.2 – 49.0)	0.123
CD8⁺ memory T cells (%)	27	81.3 (74.2 – 92.2)	10	67.5 (51.0 – 85.8)	0.123
CD8⁺ CM T cells (%)	27	5.9 (2.6 – 9.4)	10	14.7 (7.5 – 19.7)	**0.006**
CD8⁺ EM T cells (%)	27	27.0 (16.3 – 37.5)	10	27.9 (24.8 – 49.2)	0.159
CD8⁺ EMRA T cells (%)	27	42.2 (29.6 – 57.1)	10	19.7 (12.8 – 26.4)	**<0.001**
CD8⁺CD28^{null} T cells (%)	27	47.0 (35.8 – 69.1)	10	22.2 (15.1 – 32.2)	**<0.001**
CD8⁺CD28⁺CD27⁺ (%)	28	47.9 (29.5 – 62.1)	10	76.1 (70.3 – 85.0)	**<0.001**
CD8⁺CD28^{null}CD27⁻ (%)	28	35.6 (26.6 – 57.9)	10	7.4 (3.7 – 11.0)	**<0.001**
CD8⁺CD28⁺PD–1⁻ (%)	28	38.7 (24.8 – 49.2)	10	57.8 (40.6 – 69.9)	**0.026**
CD8⁺CD28^{null}PD–1⁺ (%)	28	13.1 (6.9 – 18.7)	10	10.3 (6.9 – 19.5)	0.804
CD8⁺CD28⁺CD57⁻ (%)	28	49.7 (30.6 – 63.2)	10	77.3 (66.9 – 84.0)	**<0.001**
CD8⁺CD28^{null}CD57⁺ (%)	28	30.5 (21.3 – 49.1)	10	11.6 (7.9 – 14.9)	**<0.001**

Data are presented as medians (interquartile range). CMV indicates cytomegalovirus; CM, central memory, EM, effector memory; and EMRA, effector memory CD45RA⁺.

CD4⁺ T cells was also found in the lymphoid tissues of deceased pediatric and young adult organ donors in a study by Thome et al. (142). Furthermore, there was a lower frequency of highly differentiated T cells within LN samples in contrast to PB. This finding was further supported by the other T-cell memory differentiation markers (CD28, CD27, PD-1 and CD57). It is known that CD4⁺CD28^{null} are more differentiated than CD8⁺CD28^{null}, as the latter can still contain CD27 in contrast to the CD4⁺ T-cell fraction (75, 139, 140). However, both the CD8⁺CD28^{null} T cells as well as the CD8⁺CD28^{null}CD27⁻ T cells were more frequent in PB. Even though a positive correlation existed between LN and PB samples for the majority of the different T-cell subset frequencies, the highly differentiated T cells (i.e. CD28^{null} T cells) were far more abundant in PB. The low number of highly differentiated T cells in LN might be explained by the lack of co-expression of CCR7, which T cells use for homing to secondary lymphoid organs (31).

The parameters for thymic output showed similar results for both the CD31 and the TREC assay. The results indicate a remarkably high correlation between the data obtained in the circulation and within LN. In addition, CD31⁺ T cells and TREC content in LN T-cell population showed a relation with the age of the individual as we and others have described before for circulating T cells (11, 13, 18, 134, 143). Taken together it can be concluded that the loss of thymic function with increasing age is also reflected in the secondary lymphoid tissue. Primary immune responses (e.g. vaccination) are initiated within LN. The intricate relation between the amount of naive T cells in PB and LN therefore probably explains why the number of circulating naive T cells is associated with the response to vaccination (144).

In this study we did not find any differences regarding the RTL between PB and LN. This could be explained by the depth of the analysis of RTL. We took the CD4⁺ and CD8⁺ T cell-population as a whole instead of focusing on the different T-cell subsets. Because of this, differences in RTL due to differences in frequencies of subsets might be overlooked. Furthermore, we cannot fully exclude that the isolation procedure resulted in a shift in T-cell subsets, possibly influencing the analysis of the RTL.

Age-related changes were observed in both CD4⁺ and CD8⁺ T-cell compartments within the lymph node. The frequency of naive T cells declined, while the frequency of memory T cells increased. Other studies also showed an age-related decline in naive T cells within LNs of healthy individuals and from deceased organ donors (49, 142, 145). Furthermore, a study by Thome et al. found that EM T cells were less abundant in pediatric lymphoid tissues, while these were more frequent in young adult lymphoid tissues (142). Next to this, the TREC content also decreased with increasing age in the LN, supporting the T-cell ageing process in LN. These results indicate that age-related changes in T-cell composition are similar for PB and LN with the exception of the late differentiated T cells, which appear to remain outside the LN.

CMV infection dramatically changes the composition of circulating T cells and almost doubles the total number of CD8⁺ T cells, in particular with a late differentiation phenotype (27, 42, 43). However, the T-cell differentiation status within the LNs did not differ between CMV+ and CMV– patients. In a study of Remmerswaal et al., the frequency of CMV-specific CD8⁺ T cells was significantly lower in the LN compared with the PB, which is in line with our findings (146). Next to this, a study by Havenith et al. also showed the absence of highly differentiated CD4⁺CD28^{null}CD27⁻ T cells in the lymph node, which can appear due to a CMV infection (147). These findings suggest that a latent CMV infection does not have a significant impact on T cells within LN, while the opposite is true for PB T cells.

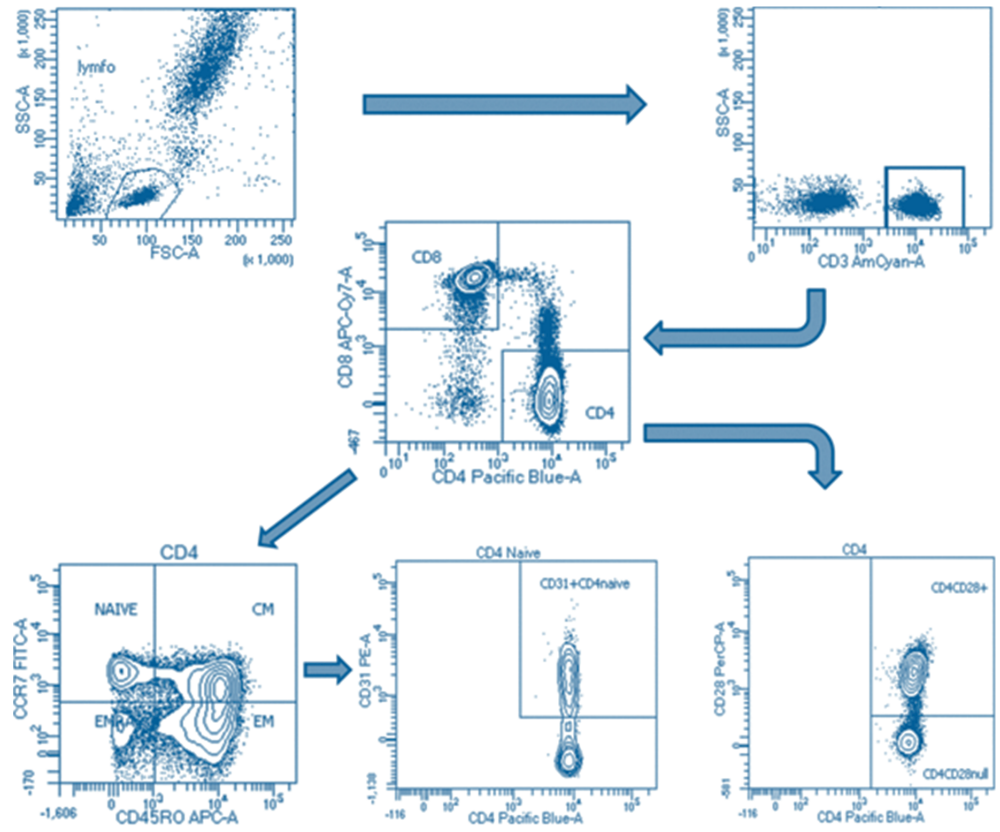
In conclusion, the T-cell composition in PB is strongly related to T-cell composition of the LN, while the presence of highly differentiated CD28^{null} T cells is more exclusive

to the PB compartment. In addition, CMV changed the T-cell differentiation status in PB drastically, while no significant alterations were observed within LN with regard to T-cell differentiation and ageing parameters. This study provides a new perspective on the T-cell immune system, especially of those with ESRD. PB seems to harbor the majority of terminally differentiated T cells, while LNs do not harbor this specific subset of T cells. These findings suggest that during an immune response the late differentiated T cells directly access the tissue and exert their immune function. This is consistent with the observation that many of these cells carry the chemokine receptor CX3CR1 for the endothelium-derived chemokine fractalkine (146). Fractalkine is considered a major player in inflammatory responses, recruiting leukocytes into the inflamed tissue (148-150). These late differentiated T cells then can respond to IL-15 produced by the inflamed parenchym, proliferate and exert their cytotoxic function (94, 95, 151). Whether the T-cell characteristics in the LN might provide a better predictive model for clinical outcomes, such as the development of (co-stimulatory blockade resistant) rejection and infection after e.g. kidney transplantation is subject to further investigation.

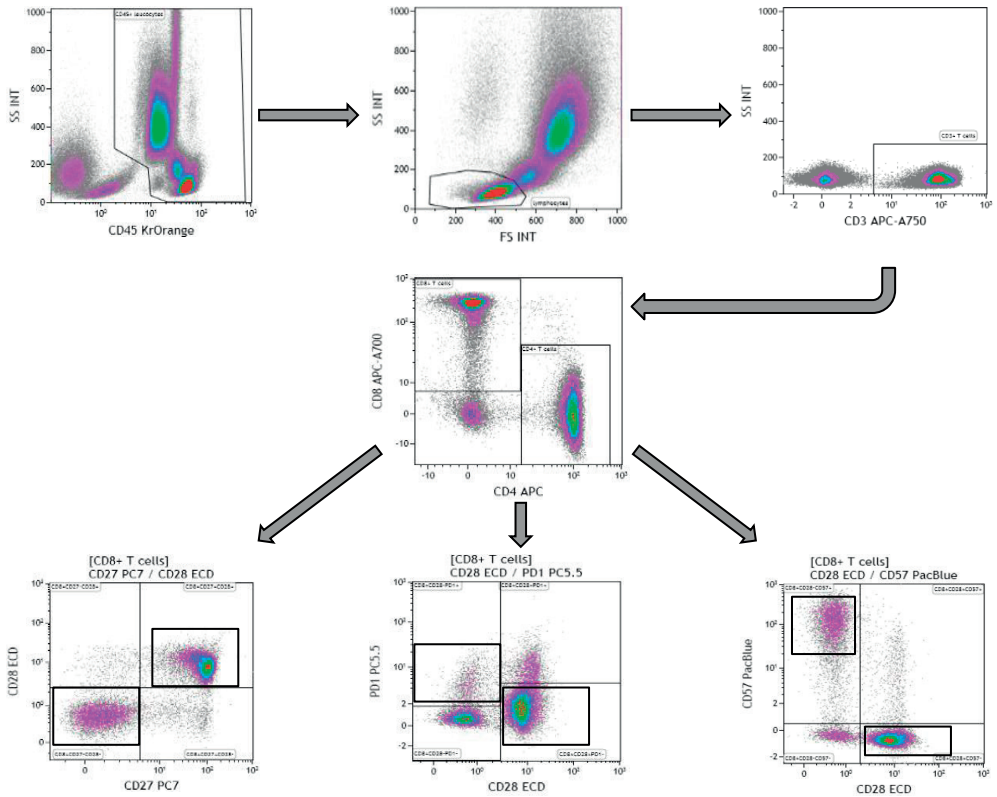
DISCLOSURES

The authors declare no competing financial and commercial interests.

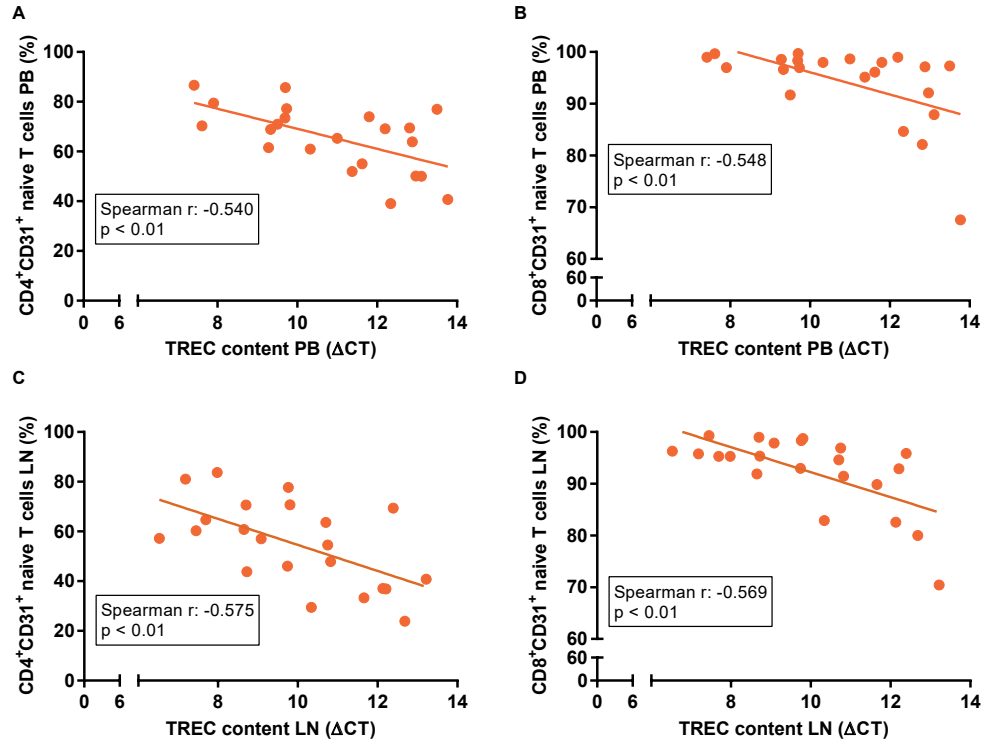
SUPPLEMENTARY FILES



Supplementary Figure 1. Gating strategy of the T-cell subsets. First, lymphocytes were selected based on the forward- and sidescatter, and $CD3^+$ T cells were selected from these lymphocytes. The $CD3^+$ T cells were then divided further into $CD4^+$ and $CD8^+$ T-cell fractions. Each population was dissected into different subsets using CCR7 and CD45RO. After this division, $CD4^+$ (only this is shown) and $CD8^+$ naive T cells were analyzed for CD31 expression. Besides this, total $CD4^+$ (only this is shown) and total $CD8^+$ T cells were analyzed for frequencies of cells lacking CD28 expression.



Supplementary Figure 2. Gating strategy of the T-cell differentiation markers. First, $CD45^+$ leucocytes were gated. From these cells, lymphocytes were selected based on the forward and side-scatter; $CD3^+$ T cells were selected from these lymphocytes. The $CD3^+$ T cells were then divided further into $CD4^+$ and $CD8^+$ T-cell fractions. Each fraction was analyzed further for the expression of CD27, programmed death 1 (PD-1) or CD57 in combination with CD28 (only the $CD8^+$ T cell fraction is shown). From these analyses only the least ($CD28^+CD27^+$, $CD28^+PD\text{-}1^-$ and $CD28^+CD57^-$) and most differentiated ($CD28^{null}CD27^-$, $CD28^{null}PD\text{-}1^+$ and $CD28^{null}CD57^+$) T cells were selected (indicated within the black frames).



Supplementary Figure 3. Correlation between $CD31^+$ naive T cells and the T-cell receptor **excision circles (TREC) content in peripheral blood (PB) and the lymph node (LN).** The Spearman's rho correlation analysis is shown between $CD4^+CD31^+$ naïve T cells within the PB and the TREC content within the PB (A), between $CD8^+CD31^+$ naive T cells within the PB and the TREC content within the PB (B), between $CD4^+CD31^+$ naive T cells within the LN and the TREC content within the LN (C) and between $CD8^+CD31^+$ naive T cells within the LN and the TREC content within the LN (D). Frequencies of cells are depicted on the y-axis, and the ΔCT which is related inversely to the TREC content is depicted on the x-axis.

Chapter 6

T-CELL COMPOSITION OF THE LYMPH NODE IS ASSOCIATED WITH THE RISK FOR EARLY REJECTION AFTER RENAL TRANSPLANTATION

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ABSTRACT

Background

The T-cell composition within the lymph node (LN) of end-stage renal disease (ESRD) patients differs from the composition within the circulation. Activation of the alloreactive T-cell response within secondary lymphoid organs is important after organ transplantation. However, to date no data are present on lymph node T cell subsets and the risk for acute rejection after kidney transplantation.

Methods

T cells from LNs of ESRD patients were analyzed for frequency of recent thymic emigrants, relative telomere length, expression of differentiation markers, and were related to the development of early acute rejection (EAR), occurring within three months after renal transplantation (RT). Furthermore, the alloreactive potential of mononuclear cells isolated from the LN and peripheral blood of 10 patients was analyzed. Measures of alloreactive potential included proliferation, cytokine production, frequencies of IFN-γ producing cells and the presence of cytotoxic molecules.

Results

Patients with EAR were younger (p=0.019), CMV-seropositive (p=0.037) and usually received dialysis prior to RT (p=0.030). Next to this, patients with EAR showed a lower CD4:CD8 ratio (p=0.027) within the LN. T cells from the LN were similar with regard to alloreactive capacity compared to those within the circulation. Univariate regression analysis showed that the CD4:CD8 ratio (OR: 0.67, p=0.039), patient age (OR: 0.93, p=0.024) and preemptive RT (OR: 0.11, p=0.046) were associated with EAR. After a multivariate analysis only the CD4:CD8 ratio (OR: 0.58, p=0.019) and preemptive RT (OR:0.05, p=0.012) were associated with EAR.

Conclusion

A lower CD4:CD8 ratio in the LN is associated with a higher risk for the development of rejection within three months after RT.

INTRODUCTION

The composition and function of the T-cell compartment in patients prior to renal transplantation (RT) may affect the risk for subsequent acute rejection and infection after transplantation (10, 35, 36, 66, 124, 141, 152). For instance, ageing-related expansion of highly differentiated T cells within PB, such as $CD8^+$ EMRA T cells or $CD4^+CD28^{null}$ T cells, is associated with a lower risk for early acute rejection after RT (36, 66).

However, T cells within PB are only one of the cell populations that play a role in allograft rejection. Another compartment which may have a significant role in the development of acute rejection is the lymph nodes (LNs). Early after organ transplantation, the direct pathway of alloantigen recognition plays the most important part in the initiation of the alloreactive response (153). One of the key events for this response is migration of donor-derived dendritic cells to LNs of the host, which is followed by activation of alloreactive T cells (153, 154). Next to this, a semi-direct pathway is also being described (153, 155), which involves transfer of intact MHC:peptide complexes from donor antigen presenting cells (APCs) to host APCs, and simultaneous presentation of the processed antigens to $CD4^+$ T cells in an indirect manner and the intact complexes to $CD8^+$ T cells in a direct manner (155, 156). These pathways show the importance of the T-cell composition within LNs with regard to development of rejection.

The T-cell composition of LN differs substantially from peripheral blood (PB) as T-cell subsets may or may not be able to migrate to LNs. We and others have studied the T-cell composition of the LN of ESRD patients and found that highly differentiated T cells were virtually absent from LNs, while they were present within PB (146, 157). Whether the LN T-cell composition and alloreactivity is associated with development of acute rejection after RT has not been studied thus far.

Therefore, in this study we investigated whether phenotypical T-cell characteristics within LNs showed an association with development of rejection within 3 months after RT. Next to this, we have also investigated functional differences between LN and PB, to assess whether there were variations with regard to alloreactive potential between these two compartments. These possible functional differences were studied by performing mixed lymphocyte reactions (MLRs) and analyzing proliferation, cytokine production and presence of cytotoxic molecules within supernatants.

MATERIALS AND METHODS

Study population

Patients prior to RT in the period from August 2015 to March 2016 were suitable for participation. Each patient gave written informed consent to take part in this study. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2015-301) and was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Clinical variables were assessed as shown in Table 1, including age of the recipient and donor, gender, CMV-seropositivity, human leukocyte antigen (HLA) class I and

class II mismatches, current and historical panel reactive antibody (PRA) scores, number of RTs, warm ischemia time, number of living donor transplants, cause of chronic kidney disease, preemptive RT (defined as receiving a kidney before initiation of renal replacement therapy), number of related RT (receiving a kidney from a genetically related donor) and type of rejection. The HLA-typing was assessed according to international standards (American Society for Histocompatibility and Immunogenetics/the European Federation for Immunogenetics) using serologic and DNA-based techniques. The PRAs were determined at the laboratory of the blood bank in Leiden, the Netherlands.

Early acute rejection (EAR) was defined as development of biopsy-proven acute allograft rejection according to the Banff criteria (158, 159) within 3 months after RT.

PBMC and LNMC isolation

Peripheral blood mononuclear cells (PBMCs) and lymph node mononuclear cells (LNMCs) were isolated as described previously (157). The isolated PBMCs and LNMCs were stored at -150°C with a minimum amount of 10×10^6 cells/vial. Median yield of the lymph node amounted to 20×10^6 cells.

T-cell differentiation status

Whole blood and freshly isolated LNMCs were used to assess T-cell differentiation status by flowcytometry. Differentiation status was based upon the study by Sallusto et al. (22), as described in detail previously (11). Briefly, expression of CD45RO and CCR7 was used to determine naive and memory T-cell populations. Memory T-cell subsets were further defined as central memory (CM) T cells ($CD45RO^+CCR7^+$ T cells), effector memory (EM) T cells ($CD45RO^+CCR7^-$ T cells) and EMRA T cells ($CD45RA^+CCR7^-$ effector memory T cells) (22). In addition, CD28 expression was analyzed as this is lost upon T-cell differentiation (20, 23, 27, 29). Cells were stained as described previously (36), and were measured on the FACSCanto II (BD) and analyzed with the FACS Diva software version 6.1.2 (BD).

To dissect T cells into early and late differentiated cells, expression of CD28, CD27, CD57 and PD-1 was also analyzed. Naive and central memory T cells express CD28 and CD27. Effector memory and EMRA T cells progressively lose expression of these molecules ($CD4^+$ T cells first lose CD27 and then CD28, while the opposite is true for $CD8^+$ T cells (75, 139, 140)) and express CD57 and PD-1 (24-26, 138). Thus, early differentiated T cells were defined as $CD28^+CD27^+$, $CD28^+PD\text{-}1^-$ and $CD28^+CD57^-$ T cells, while $CD28^{null}CD27^-$, $CD28^{null}PD\text{-}1^+$ and $CD28^{null}CD57^+$ T cells were defined as late differentiated T cells. This measurement was performed on the Navios flow cytometer (Beckman Coulter) and collected data were evaluated with the Kaluza software version 1.3 (Beckman Coulter). T cells were analyzed with DuraClone IM T-cell subsets tubes (Beckman Coulter) as described previously (157).

Relative Telomere Length

Relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells was determined by using flow fluorescent in situ hybridization on thawed PBMCs and LNMCs, as described in detail previously (11).

Assessment of recent thymic emigrants using CD31 and TREC content

Recent thymic emigrants (RTEs) were defined as naive T cells expressing CD31 and were assessed by flow cytometry, as described previously (125). T-cell receptor excision circles (TREC) content was determined using 1×10^6 snap-frozen PBMCs and LNMCs. DNA was isolated from these snap-frozen samples and TREC content was detected using quantitative PCR as described previously (13). The TREC content is depicted as 1/DCT.

Allogeneic stimulation

PBMCs and LNMCs from renal transplant recipients (responders) were thawed and rested overnight. Then PBMCs and LNMCs were labeled with CFSE (Molecular Probes®, Leiden, the Netherlands) according to manufacturer's instructions and stimulated in triplicate at $5x10^4$/well with irradiated PBMCs (40 Gy) of their corresponding donor, at a 1:1 ratio for 6 days. As a negative control, responders were stimulated with their own irradiated PBMCs or LNMCs (autologous stimulation). Responder cells were stimulated with phytohaemagglutinin (PHA) 5 µg/ml to examine their maximum proliferative potential. On day 6, wells of the same condition were pooled and supernatant stored at -80°C. Proliferation was analyzed by measuring CFSE dilution and determining the frequency of $CFSE^-$ cells. For this purpose, cells were stained using the following antibodies; AmCyan-labeled anti-CD3 (BD), pacific blue (PacB)-labeled anti-CD4 (BD), APC-Cy7-labeled anti-CD8 (BD), phycoerythrin (PE)-Cy7-labeled anti-CCR7 (BD Pharmigen), APC-labeled anti-CD45RO (BD), PE-labeled anti-CD28 (BD). A dump-channel was applied to exclude unwanted cells from the analysis, by co-staining cells for the live-dead marker 7-AAD, peridin chlorophyll protein (PerCP)-labeled anti-CD19 (BD), PerCP-Cy5.5-labeled anti-CD56 (Biolegend) and PerCP-labeled anti-CD14 (BD) (Supplemental figure 1). Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD).

Analysis of cytokine and granzyme B production

Concentrations of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and granzyme B were determined from collected supernatants. These supernatants were analyzed with the human cytometric bead array (CBA) flex set (BD) according to manufacturer's instructions. Briefly, a standard curve for each analyte using a 4-parameter logistic regression analysis was created. This curve was based upon standards with fixed concentrations of each analyte and their corresponding median fluorescence intensities (MFIs). Then, MFI of the various analytes within the samples were converted to concentrations (pg/mL). Samples were

measured on the FACS Canto II (BD) and concentrations were determined with GraphPad Prism 5 (CA, USA).

IFN-γ ELISPOT assay

Frequencies of IFN-γ producing cells (spots/100.000 cells) following autologous, allogeneic or PHA stimulation were measured with an Enzyme-Linked ImmunoSpot (ELISPOT) assay (U-CyTech, Utrecht, The Netherlands). During the first day, the ELISPOT plate was coated with the antibody and incubated overnight. The same day cells were thawed and rested overnight. The following day, the assay plate was blocked using a blocking buffer and incubated for 1 hour at 37°C. After the plate was washed with phosphate buffered saline (PBS), cells were pipetted into wells and stimulated in triplicate, as described earlier, for 1 day. Thereafter, plates were washed first with PBS and then with PBS-Tween. Spots were made visible according to manufacturer's instructions. Spots were analyzed using the ELISpot reader (Bioreader®-600V, BIO-SYS GmbH, Karben, Germany).

Statistical analysis

Variables are presented as medians with interquartile ranges. Differences between paired samples (PB and LN from the same patient) were analyzed using the Wilcoxon signed-rank test. Differences between continuous variables from two independent groups were assessed with the Mann–Whitney U test. Differences between categorical variables were analyzed either with the Pearson's chi-squared test or with the Fisher's exact test depending on the expected values in any of the cells of a contingency table. Associations between rejection and the assessed parameters were analyzed using a binary logistic regression analysis. The significance level (p-value) was two-tailed and an α of 0.05 was used. Statistical analyses were performed using SPSS® version 21.0 for Windows® (SPSS Inc., IL, USA) and GraphPad Prism 5 (CA, USA). Figures were created with GraphPad Prism 5 (CA, USA).

RESULTS

Patient characteristics

Patient characteristics (*n*=38) are shown in Table 1. Median patient age was 58 years and median donor age was 55 years. Most of the patients underwent an RT for the first time (82%), while 18% underwent a transplantation for the second time. Most common cause of ESRD was nephrosclerosis/atherosclerosis/hypertension (29%) followed by polycystic kidney disease (21%), together accounting for half of the cases. Overall, 24 (63%) patients received dialysis treatment prior to RT. Eleven of the 38 patients (29%) developed an EAR. Majority of the rejections were classified as cellular rejection (64%) and the remaining as antibody-mediated rejection. Median patient age was significantly younger within the rejectors compared with the non-rejectors (47 vs 60, p<0.019). All patients who developed an EAR were CMV-seropositive, while 63% of the non-rejectors was CMV-seropositive (p=0.037). Median warm ischemia time was longer for the rejectors compared with the non-rejectors (23 minutes vs 17 minutes, p=0.045). Majority of the rejectors had dialysis prior to RT (91%), while 52% of the non-rejectors received this therapy (p=0.030). Immunological risk factors like PRA score, HLA mismatches or unrelated donor transplantation were not different between the two groups.

Table 1. Patient Characteristics

RT Patients (*n*=38)	No Rejection (n=27) (71%)	Rejection (*n*=11) (29%)	p
Age recipient	60 (51 – 66)	47 (37 – 58)	**0.019**
Age Donor	51 (42 – 61)	63 (50 – 69)	0.129
Male gender recipient	17 (63%)	7 (64%)	>0.999
CMV-seropositivity recipient	17 (63%)	11 (100%)	**0.037**
CMV-serostatus donor/recipient			
-/-	6 (29%)	0 (0%)	0.154
-/+	7 (26%)	7 (64%)	0.061
+/-	4 (15%)	0 (0%)	0.303
+/+	10 (37%)	4 (36%)	>0.999
Mismatch HLA class I	3 (2 – 4)	3 (2 – 4)	0.727
Mismatch HLA class II	1 (1 – 2)	1 (1 – 2)	0.824
Mismatch HLA class I and II	4 (4 – 5)	5 (3 – 5)	0.949
PRA current (%)	0 (0 – 4)	0 (0 – 33)	0.446
PRA historic (%)	4 (0 – 29)	6 (0 – 73)	0.727
Number of RT	1 (1 – 1)	1 (1 – 1)	>0.999
Warm ischemia time (minutes)	17 (13 – 22)	23 (20 – 27)	**0.045**
Living donor transplant	22 (81%)	8 (73%)	0.667
Cause of CKD			
Nephrosclerosis/atherosclerosis/ hypertension	6 (22%)	5 (45%)	0.238
Primary glomerulopathies	3 (11%)	2 (13%)	0.615
Diabetes	7 (26%)	0 (0%)	0.084
Polycystic Kidney Disease	6 (22%)	2 (18%)	>0.999
Other	2 (7%)	1 (9%)	>0.999
Unknown	3 (11%)	1 (9%)	>0.999
Pre-emptive RT	13 (48%)	1 (9%)	**0.030**
Genetically-related RT	1 (4%)	1 (9%)	0.501
Acute Rejection type			
Cellular rejection		7 (64%)	
Antibody-mediated rejection		4 (36%)	

Data are presented as medians (interquartile range) or as numbers (percentages). CKD indicates chronic kidney disease; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibody; RT, renal transplantation.

T-cell composition of the LN prior to RT shows an association with EAR

Figures 1 and 2 show the T-cell differentiation status of $CD4^+$ and $CD8^+$ T cells within the LNs of the rejectors and non-rejectors. Median frequency of $CD4^+$ T cells prior to RT is significantly lower (p=0.014) and median frequency of $CD8^+$ T cells is significantly higher (p=0.019) within the rejectors (Figures 1A and 1B). This led to a significantly lower CD4:CD8 ratio in LNs of patients with EAR (3.4 vs 5.7, p=0.027) (Figure 1C). Furthermore, median frequencies of $CD4^+$ EM T cells and $CD4^+CD28^{null}$ T cells were significantly higher within the rejectors (p=0.044 and p=0.008, respectively) (Figures 2C and 2G). But overall, median percentage of $CD4^+CD28^{null}$ T cells remained low in both groups (0.8% vs 1.5%). No other significant differences were observed for the other $CD4^+$ or $CD8^+$ T-cell subsets.

Assessment of T-cell differentiation status can be further refined by differential expression levels of CD27, CD57 and PD-1 in combination with CD28. However, this approach did not show any differences between the two groups (Table 2).

In summary, a lower CD4:CD8 ratio was associated with rejection.

RTL, RTEs and TREC content in the LN are not associated with rejection

The telomere length is indicative of T cell replicative history, while CD31 expression on naïve T cells and TREC content are a measure of thymic output of naïve T cells. These parameters are strongly associated with the age and therefore a measure of the biological age of the T-cell system (16-18, 45, 46, 133). Figure 3 demonstrates the RTL of T cells, RTE frequency and the TREC content in LNs between the rejectors and non-rejectors. RTL of $CD4^+$ and $CD8^+$ T cells was similar between the two groups (Figures 3A and 3B). Also, no differences were observed for $CD4^+$ and $CD8^+$ RTEs between rejection and no rejection (Figures 3C and 3D). Finally, the TREC content was also not associated with development of rejection (Figures 3E).

A lower CD4:CD8 ratio within the LN and dialysis prior transplantation are associated with rejection

Table 3 shows a univariate regression analysis of the significant clinical and immunological parameters with development of EAR. This univariate analysis showed that a higher CD4:CD8 ratio was associated with a lower risk for EAR (OR=0.67, p=0.039). Next to this, an older patient age and preemptive RT were also associated with a lower risk for rejection (OR=0.93, p=0.024; OR=0.11, p=0.046, respectively). Then these parameters were put into a multivariate regression analysis for a first model (Table 4). This showed that age was not associated with development of EAR. On the other hand, a higher CD4:CD8 ratio showed a tendency for a lower risk for rejection (OR=0.63, p=0.053), while preemptive RT still showed a significant association with development of EAR (OR=0.06, p=0.023). Taking the parameter 'age' out of the equation, showed a second model with significant associations (Table 4). A higher CD4:CD8 ratio and preemptive RT were both associated with a lower risk for EAR (OR=0.58, p=0.019; OR=0.05, p=0.012, respectively).

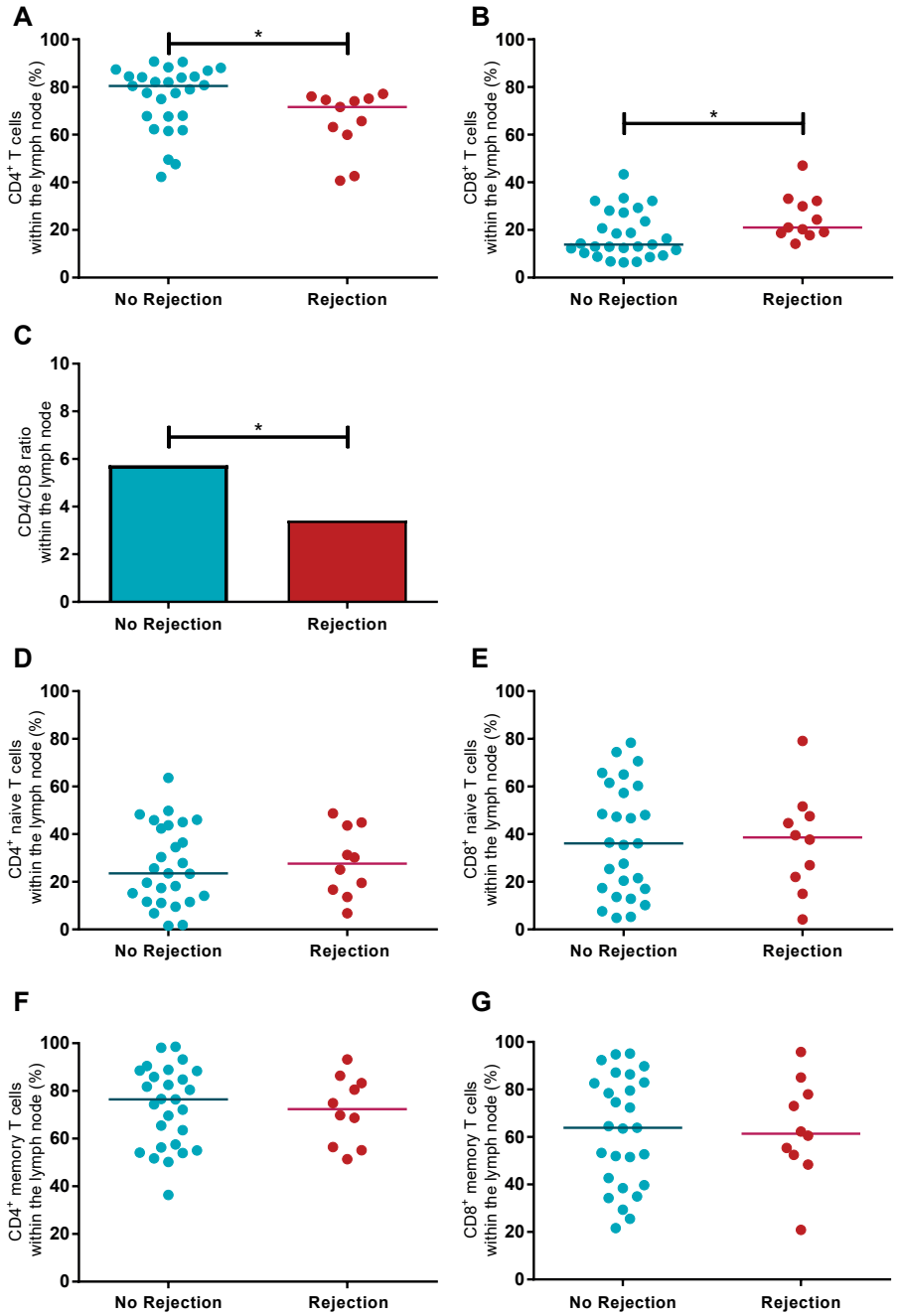


Figure 1. $CD4^+$ and $CD8^+$ T-cell subsets in the lymph node between rejection and no rejection groups. (A) Frequencies of $CD4^+$ T cells and (B) $CD8^+$ T cells, (C) CD4:CD8 ratio, (D) frequencies of $CD4^+$ naïve T cells, (E) $CD8^+$ naïve T cells, (F) $CD4^+$ memory T cells and (G) $CD8^+$ memory T cells within the lymph nodes are shown between the no rejection and rejection groups. Data are presented with individual percentages and medians or bars and medians. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

Thus, a lower CD4:CD8 ratio in the LN and receiving dialysis prior to transplantation increases the risk for the development of rejection after transplantation.

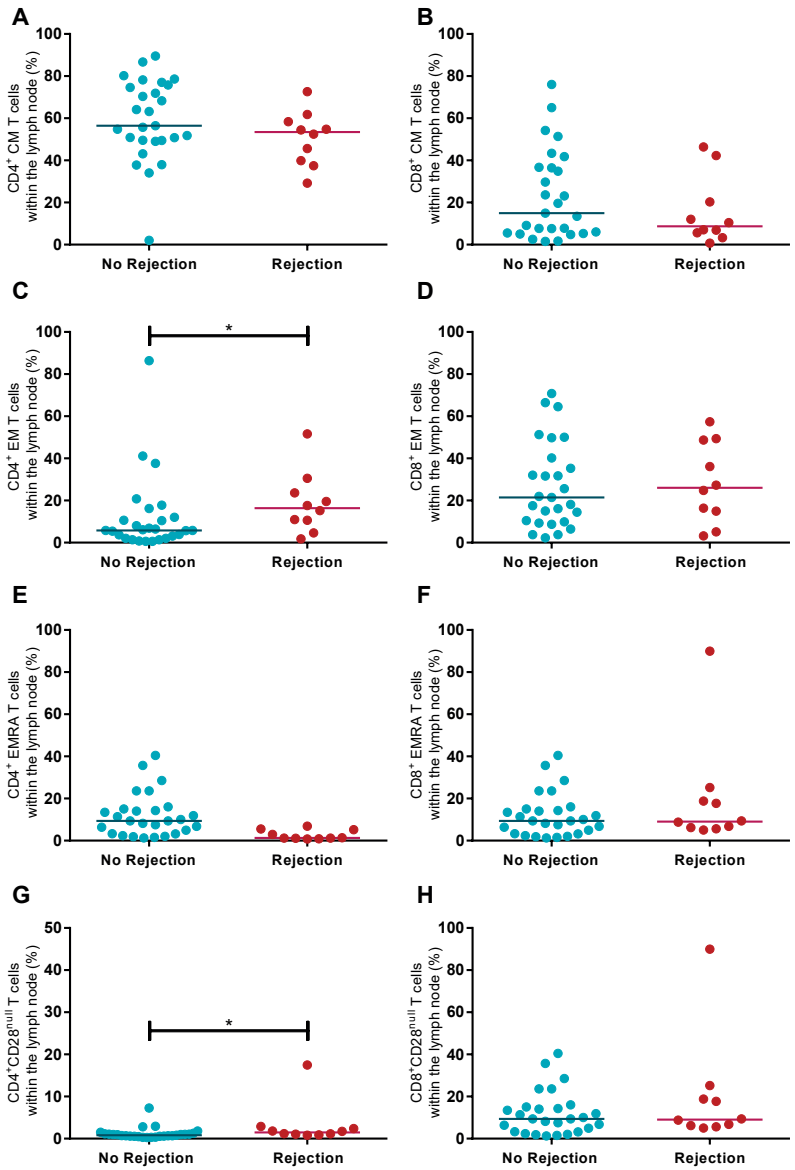


Figure 2. Memory CD4⁺ and CD8⁺ T-cell subsets and CD28 expression in the lymph node between rejection and no rejection groups. (A) Frequencies of CD4⁺ central memory (CM) T cells, (B) CD8⁺ CM T cells, (C) CD4⁺ effector memory (EM) T cells, (D) CD8⁺ EM T cells, (E) CD4⁺ effector memory CD45RA⁺ (EMRA) T cells, (F) CD8⁺ EMRA T cells, (G) $CD4^+CD28^{null}$ T cells and (H) $CD8^+CD28^{null}$ T cells within the lymph nodes are shown between the no rejection and rejection groups. Data are presented with individual percentages and medians. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

Table 2. CD4⁺ & CD8⁺ T-cell differentiation status in lymph node with regard to the development of rejection

	n	No Rejection	*n*	Rejection	p
CD4⁺					
$CD4^+CD28^+CD27^+$ (%)	25	93.8 (91.6 – 96.6)	8	93.8 (81.1 – 94.7)	0.578
$CD4^+CD28^{null}CD27^-$ (%)	25	0.3 (0.1 – 0.6)	8	0.2 (0.1 – 0.8)	0.951
$CD4^+CD28^+PD\text{–}1^-$ (%)	25	73.5 (64.0 – 82.0)	8	76.3 (72.0 – 80.4)	0.665
$CD4^+CD28^{null}PD\text{–}1^+$ (%)	25	0.4 (0.2 – 0.9)	8	0.5 (0.3 – 1.2)	0.578
$CD4^+CD28^+CD57^-$ (%)	25	97.6 (95.3 – 98.6)	8	97.0 (96.5 – 97.7)	0.272
$CD4^+CD28^{null}CD57^+$ (%)	25	0.1 (0.0 – 0.2)	8	0.1 (0.0 – 0.2)	0.885
CD8⁺					
$CD8^+CD28^+CD27^+$ (%)	25	89.6 (84.1 – 93.3)	8	90.4 (78.8 – 92.2)	0.789
$CD8^+CD28^{null}CD27^-$ (%)	25	1.9 (1.0 – 2.6)	8	1.7 (1.0 – 6.5)	0.789
$CD8^+CD28^+PD\text{–}1^-$ (%)	25	62.2 (49.5 – 75.1)	8	66.9 (45.8 – 75.1)	0.853
$CD8^+CD28^{null}PD\text{–}1^+$ (%)	25	2.4 (1.6 – 5.2)	8	2.6 (1.6 – 4.8)	0.951
$CD8^+CD28^+CD57^-$ (%)	25	87.4 (80.2 – 91.0)	8	86.3 (75.5 – 88.8)	0.374
$CD8^+CD28^{null}CD57^+$ (%)	25	1.5 (1.0 – 2.3)	8	1.5 (0.9 – 4.3)	0.757

Data are presented as medians (interquartile range). CM indicates central memory; EM, effector memory; and EMRA, effector memory CD45RA⁺.

T cells from the LN and PB proliferate similarly upon allogeneic stimulation

Median frequency of proliferating CD4⁺ T cells from the LN rose significantly from 2.4% to 45.0% (p=0.002) upon allogeneic stimulation (Figure 4A), and that of CD4⁺ T cells from the PB increased from 2.1% to 39.3% (p=0.004) (Figure 4B). Within the proliferating CD4⁺ T cells, majority of the cells were memory T cells (mainly CM) (Figures 4A and 4B). The CD28⁺ T-cell fraction was the main proliferating fraction within proliferating CD4⁺ T cells in PB and LN (>99.0% proliferation in both compartments) (Figures 4A and 4B).

The CD8⁺ T cells showed similar results compared to the CD4⁺ T cells. Median frequency of proliferating CD8⁺ T cells from the LN rose significantly from 1.4% to 22.9% (p=0.004) upon allogeneic stimulation (Figure 4C) and median frequency of CD8⁺ T cells from PB increased from 0.6% to 29.9% (p=0.002) (Figure 4D). CD8⁺ memory T cells comprised the largest fraction within proliferating CD8⁺ T cells in both LN and PB, with CM T cells also being the major contributor within this fraction (Figures 4C and 4D). The CD28⁺ T-cell fraction was again the main proliferating fraction within proliferating CD8⁺ T cells in LN and PB (Figures 4C and 4D).

Comparing the frequency of proliferating T cells upon alloreactive stimulation between LN and PB, showed no differences except for $CD8^+CD28^{null}$ T cells within proliferating CD8⁺ T cells. Median frequency of these cells within LN amounted to 0.3% while this was 2.9% within PB (p=0.037), which is probably due to the higher presence of these cells within PB compared with LN.

Polyclonal stimulation with PHA induced >95% proliferation of CD4⁺ T cells and CD8⁺ T cells from both compartments, although, this response was significantly higher in CD4⁺ and CD8⁺ T cells from LN (p=0.037 for both subsets). The functional assays could not be performed in all patients as the yield of cells from the LN was not sufficient enough in some cases. For this reason the number of patients with EAR included in this part of the study was too low (*n*=3) for adequate comparison with patients without EAR.

In conclusion, the alloreactive potential is not different between T cells from LN and PB, but T cells from LN show a slightly higher proliferative potential in response to PHA.

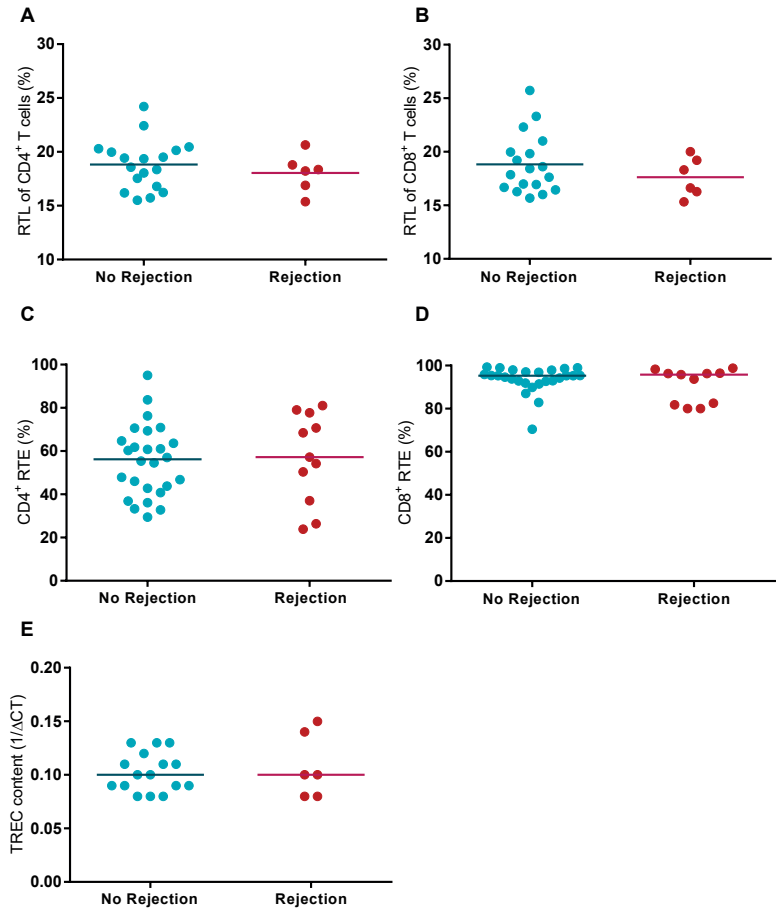


Figure 3. Relative telomere length, recent thymic emigrant frequency and T-cell receptor excision circle content in the lymph node between rejection and no rejection groups. The relative telomere length (RTL) of (A) CD4⁺ T cells and (B) CD8⁺ (T cells) within the lymph nodes between the no rejection and rejection groups are shown. Frequencies of (C) CD4⁺ and (D) CD8⁺ recent thymic emigrants (RTEs) within the lymph nodes between the no rejection and rejection group are shown. (E) The TREC content (1/ΔCT) within the lymph nodes between the no rejection and rejection group is shown. Data are shown as individual points and medians. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

Table 3. Univariate regression analysis between rejection and immunological and clinical parameters

	OR	95% CI	p
CD4⁺ T cells (%)	0.95	0.90 – 1.00	0.062
CD4⁺ EM T cells (%)	1.02	0.98 – 1.06	0.314
CD4⁺CD28^{null} T cells (%)	1.30	0.86 – 1.95	0.216
CD4:CD8 ratio	0.67	0.45 – 0.98	**0.039**
CD8⁺ T cells (%)	1.08	1.00 – 1.16	0.057
Age recipient	0.93	0.88 – 0.99	**0.024**
CMV-seropositivity recipient	0.00	0.00 – N/A	0.999
Warm ischemia time (minutes)	1.06	0.96 – 1.16	0.241
Pre-emptive RT	0.11	0.01 – 0.96	**0.046**

EM indicates effector memory; RT, renal transplantation; and N/A, not available.

Table 4. Multivariate regression analysis between rejection and immunological and clinical parameters

	OR	95% CI	p
Model 1			
CD4:CD8 ratio	0.63	0.39 – 1.01	0.053
Age recipient	0.97	0.90 – 1.04	0.393
Pre-emptive RT	0.06	0.01 – 0.68	**0.023**
Model 2			
CD4:CD8 ratio	0.58	0.36 – 0.92	**0.019**
Pre-emptive RT	0.05	0.00 – 0.51	**0.012**

EM indicates effector memory; RT, renal transplantation; and N/A, not available.

IFN-γ ELISPOT shows no differences between LN and PB, but IFN-γ and granzyme B production by LNMCs is lower than PBMCs after 6 days of allogeneic stimulation

Frequency of IFN-γ producing LNMCs increased significantly after allogeneic stimulation (p=0.034), while a similar tendency was seen for PBMCs (p=0.083). However, median amount of spots remained low after allogeneic stimulation (3.5/1×10⁵ for the LN and 6/1×10⁵ for the PB). Furthermore, there were no significant differences between the two different compartments after allogeneic stimulation (Figure 5A). Stimulation with PHA, showed a significantly higher IFN-γ secretion by PBMCs compared with T cells within LNMCs (256/1×10⁵ vs 126/1×10⁵, p=0.047).

After allogeneic stimulation of LNMCs and PBMCs for 6 days, concentrations of TNF-α, IFN-γ and granzyme B in the supernatants were significantly increased. LNMCs had a lower production of TNF-α (p=0.065) (Figure 5B), IFN-γ and granzyme B compared with PBMCs (p=0.020 and p=0.049, respectively) (Figures 5C and 5D). Interestingly, amount of IFN-γ spots after allogeneic stimulation for 1 day correlated significantly with

production of IFN-γ by LNMCs as well as PBMCs after 6 days (ρ=0.88, p=0.001 and ρ=0.64, p=0.048, respectively).

Thus, production of TNF-α, IFN-γ and granzyme B by LNMCs and PBMCs increases after allogeneic stimulation. However, generation of these products is lower by LNMCs than by PBMCs.

DISCUSSION

This study has investigated for the first time the relation between T-cell characteristics of the lymph node and acute rejection after RT. Surprisingly, a lower CD4:CD8 ratio in the LN was significantly associated with a higher risk for rejection. Studies have shown that CD8+ T cells play an important role in allograft rejection (160, 161). Antigen presenting cells can present the alloantigen to CD4+ and CD8+ T cells that are present within the lymph node, which will lead to the activation of these cells. Having a lower CD4:CD8 ratio might tip the balance towards a more cytotoxic profile, which could promote allograft rejection.

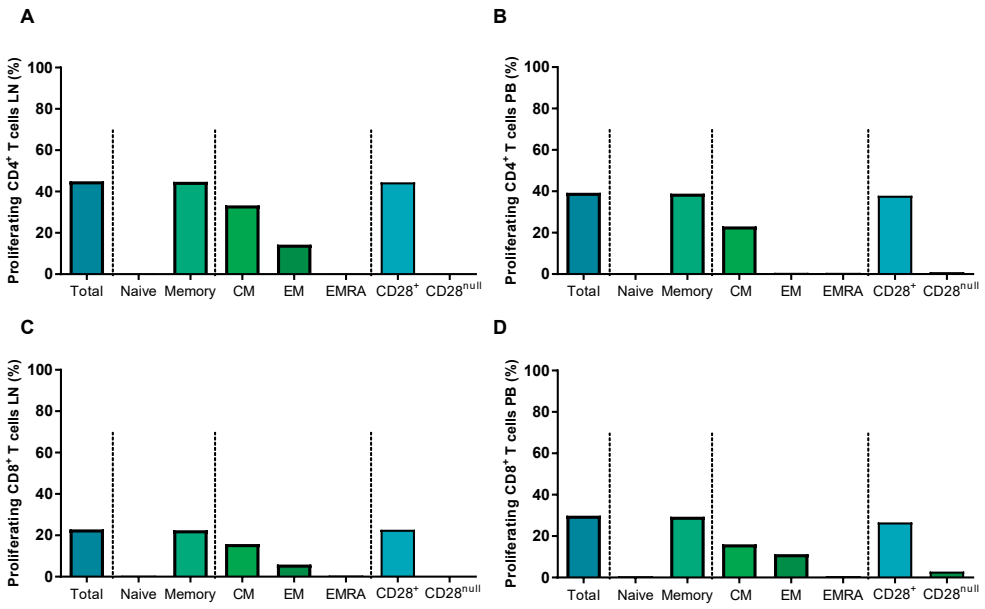


Figure 4. Proliferation of CD4+ and CD8+ T cells from the lymph node and peripheral blood after allogeneic stimulation. Frequencies of proliferating CD4+ T cells within (A) the lymph node and (B) the peripheral blood are shown after allogeneic stimulation. Frequencies of proliferating CD8+ T cells within (C) the lymph node and (D) the peripheral blood are shown after allogeneic stimulation. Each graph is divided into 4 parts marked with the dotted lines. From left to right; the total proliferating T-cell population is shown, which then is subdivided into naïve and memory T cells in the second portion, the third portion shows the subdivision of the memory T-cell population into central memory (CM), effector memory (EM) and effector memory CD45RA+ (EMRA) T cells, the fourth portion shows the subdivision of the total T-cell population into CD28+ and CD28null T cells. Frequencies are presented with bars and medians.

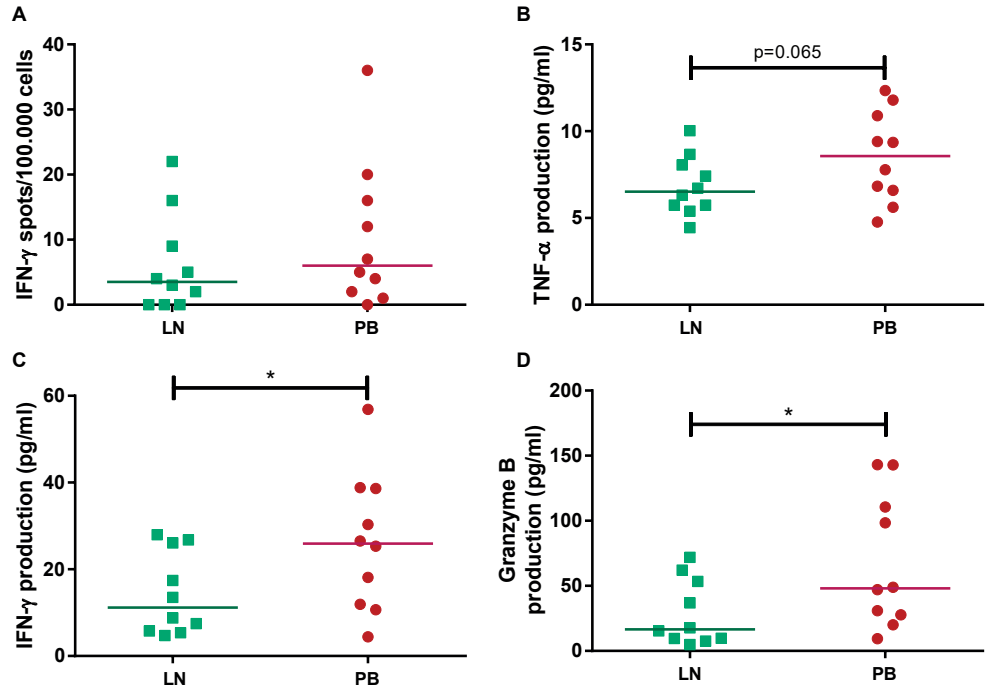


Figure 5. IFN-γ, TNF-a and Granzyme B production after allogeneic stimulation. (A) The number of IFN-γ spots per 100.000 cells by lymph node mononuclear cells (LNMCs) and peripheral blood mononuclear cells (PBMCs) after allogeneic stimulation for 1 day is shown. The production of (B) TNF-α, (C) IFN-γ and (D) granzyme B (pg/ml) by LNMCs and PBMCs after allogeneic stimulation for 6 days is shown. Data are shown as individual points and medians. Significant differences were calculated and shown (*p<0.05, **p≤0.01, ***p≤0.001).

A study by Ford et al. has shown in a murine skin graft model that, a high initial frequency of antigen-specific CD8+ T cells resulted in the development of CD8+ T cells which were able to produce multiple cytokines and were more prone to escape co-stimulation blockade (162). Next to this, a study by Shenoy et al. showed that a lower CD4:CD8 ratio in bronchus-associated lymphoid tissue was associated with a higher frequency of acute rejections within one year after lung transplantation (163). Thus, having a high initial frequency of CD8+ T cells in lymph nodes, could lead to a higher chance for the presence of alloreactive CD8+ T cells, increasing the risk for rejection.

It is known that alloantigen recognition can occur via direct or indirect pathways (153), but next to this a semi-direct pathway is also being described (153, 155). This semi-direct pathway involves the transfer of intact MHC:peptide complexes from donor APCs to host APCs and also the processing of antigens by the host APCs (155). The host APCs can then simultaneously present the processed antigens to CD4+ T cells in an indirect manner, while they present the intact complexes to CD8+ T cells in a direct manner (155, 156). At the same time, the CD4+ T cells can provide help to the CD8+ T cells to activate them

(155). It has been shown that CD4+ T cells which are activated by the indirect pathway, can successfully enhance direct pathway CD8+ T-cell responses (164). As the direct pathway is the most important pathway in the initiation of alloreactive responses (153), a higher frequency of CD8+ T cells within the lymph nodes prior to transplantation will increase the possibility for allorecognition by the direct and also by the semi-direct pathway.

Next to this, we have found that younger patients have a higher risk for EAR. Even though this finding was not significant after a multivariate analysis, it still showed an important difference between the rejection and no rejection groups after a univariate analysis. This association between a younger age and an increased risk for rejection has also been found by previous studies (165, 166). An aged immune system in the older patients may underlie the reduced risk for rejection. In addition, ESRD patients undergo uremia-associated premature ageing of their T-cell system (11, 13, 125), which further impairs the alloreactivity of their immune system. For this reason we have studied in detail the relation between several immune parameters of T-cell ageing and clinical outcome after RT. In particular, the increased presence of highly differentiated CD8+ EMRA T cells or $CD4^+CD28^{null}$ T cells in the circulation, was associated with a decreased risk for acute rejection but no other parameters such as telomere length and thymic output of naïve T cells (36, 66). In this study we did not find an association between all tested immune parameters of T cell ageing and acute rejection. The absence of highly differentiated T cells within the LN is most likely the reason that we also could not relate the presence of these cells to acute rejection (157).

Furthermore, we analyzed differences in IFN-γ production by mononuclear cells isolated from the LN and PB using the ELISPOT assay. Previous studies have shown that this assay shows important correlations with regard to rejection and graft function after RT (167-170). However, we saw no differences with regard to IFN-γ producing cells from both compartments. On the other hand, we saw that the production of IFN-γ and granzyme B was higher by PBMCs after 6 days of stimulation. Again, this is probably due to the higher presence of more differentiated cells within the PB compared with the LN (157). Next to this, proliferation of T cells upon stimulation with alloantigens was also similar between the two compartments. This means that LNMCs have a similar alloreactive potential, with the LNMCs showing a lower production of cytokines and cytotoxic molecules, due to the presence of less differentiated T cells within the LNs.

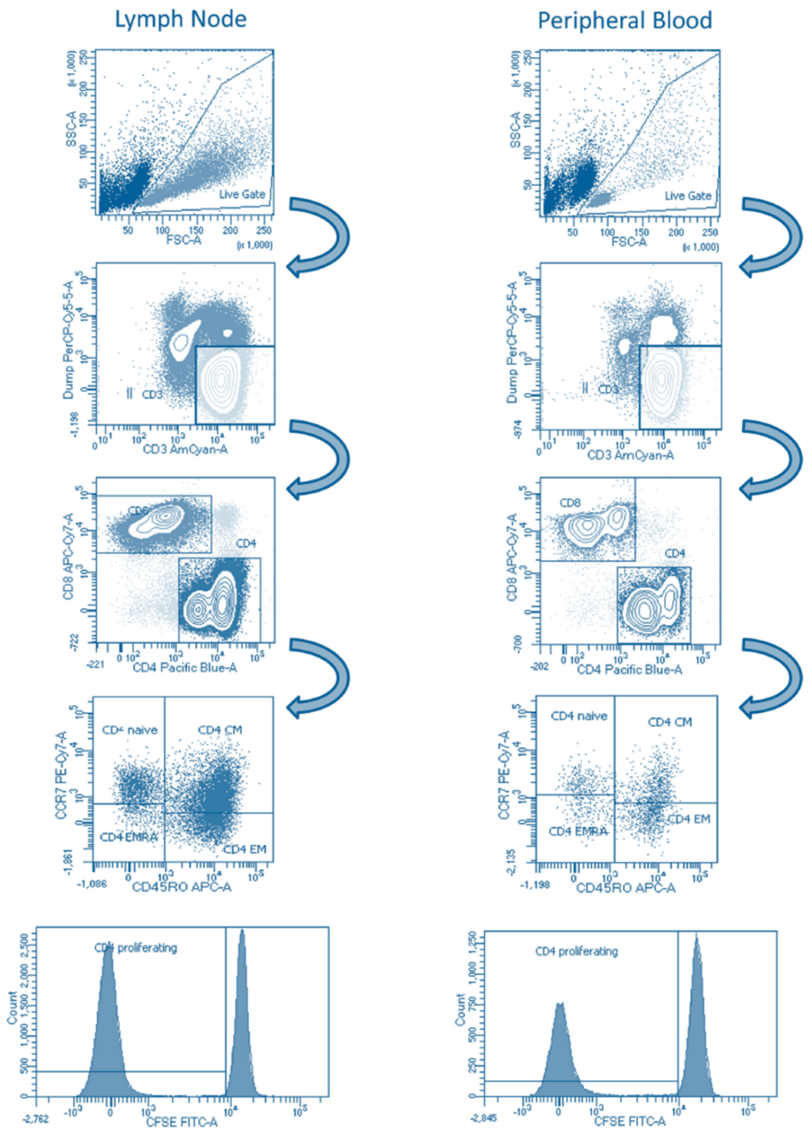
This study is the first which shows an association between the T-cell CD4:CD8 ratio of the lymph node and the development of early acute rejection after RT. Although this finding is of considerable interest, it needs to be validated in a study with a larger cohort. However, the results show that the LN should be considered as a different compartment with its own contribution to the risk for the development of rejection. Although speculative, one of the implications of our finding could be that highly differentiated memory T cells in the peripheral blood are relevant for and associated with acute rejection as they can directly enter peripheral tissues such as the renal transplant. In contrast, these cells cannot enter the LN and alloreactivity in this environment is stimulated by donor-derived APC interacting with naïve and central memory T cells. The relative contribution of both pathways needs to be further elucidated.

In conclusion, a lower CD4:CD8 ratio within LN is associated with a higher risk for the development of rejection within three months after RT.

DISCLOSURES

The authors declare no competing financial and commercial interests.

SUPPLEMENTARY FILES



Supplementary Figure 1. Analysis of proliferation after allogeneic stimulation of PBMCs and LNMCs. An example of the gating strategy is shown. The left column represents the analysis of LNMCs while the right column shows the analysis of PBMCs after allogeneic stimulation. From top to bottom; first, lymphocytes were selected based on the forward and sideward scatter. Then, living $CD3^+$ T cells were selected from these lymphocytes. The $CD3^+$ T cells were then further divided into $CD4^+$ and $CD8^+$ T cells. Each population was then further dissected into different subsets using CCR7 and CD45RO. Only $CD4^+$ T cells are shown, but the same analysis was applied on $CD8^+$ T cells. The bottom graphs show an example of the analysis of proliferation after allogeneic stimulation with CFSE dilution.

Chapter 7

SUMMARY & DISCUSSION

SUMMARY

The kidneys play an important role in several essential processes within the body. They regulate the osmolality and the volume of the extracellular fluid. This is achieved by the excretion of water, electrolytes and waste products. Next to this, the kidneys are also involved in the production of several hormones such as erythropoietin (production of erythrocytes), calcitriol/active vitamin D (bone metabolism) and renin (vasoconstriction and increase in blood pressure). Thus, a loss of renal function will have a negative impact on all the aforementioned processes.

Besides these processes, the loss of renal function also influences the immune system. Previous studies have shown that patients with end-stage renal disease undergo premature ageing of their T-cell immunity. A discrepancy of 20 to 30 years was noted between then immunological age of these patients and their calendar age. This enhanced ageing is probably initiated by the presence of uremia, which creates a proinflammatory environment. This will lead to a continuous activation of the immune system, which also involves the T cells. This process might play a part in the decreased vaccination responses and the increased susceptibility for infections, seen in end-stage renal disease patients. Thus, these changes of the immune system will also influence clinical outcomes in the setting of renal transplantation. Renal transplantation is the best option with regard to renal replacement therapy, but this also requires the use of immunosuppressive medication. These agents will reduce the risk for rejection, while on the other hand increasing the risk for infections. In patients with end-stage renal disease, who already have an impaired immune system, the use of these drugs could have a deleterious effect. Thus, predictive immunological parameters are needed to determine the risk for the development of rejection and infections after renal transplantation. This will aid in the development of personalized approaches with regard to immunosuppressive medication in renal transplantation.

In **chapter 1** the process of premature T-cell ageing in end-stage renal disease patients and the available T-cell ageing parameters are introduced. These parameters are the frequency and absolute numbers of recent thymic emigrants within naive T cells and the T-cell receptor excision circle (TREC) content, which are both measures for thymic output. Furthermore, the differentiation status of T cells and the relative telomere length of T cells, can also be used as T-cell ageing parameters. These parameters can be assessed within different compartments such as the peripheral blood and the lymph nodes.

In **chapter 2** the association between circulatory T-cell ageing parameters prior to transplantation and the risk for early acute rejection (within 3 months) after renal transplantation is discussed. Patients without an early rejection after transplantation had more $CD4^{+}CD28^{null}$ T cells prior to transplantation. A similar trend was also observed for the $CD8^{+}CD28^{null}$ T cells. None of the other parameters such as relative telomere length or the frequency of recent thymic emigrants did show an association with the risk for rejection.

In **chapter 3** the $CD4^+CD28^{null}$ are further analyzed with regard to their alloreactive potential. $CD4^+CD28^{null}$ T cells did not show any significant increase in proliferation, degranulation, cytotoxicity or cytokine production upon allogeneic stimulation without cytokines. However, addition of IL-15 (with/without IL-21) significantly increased alloreactivity. Thus, $CD4^+CD28^{null}$ T cells can become alloreactive when the optimal conditions are met.

In **chapter 4** the relationship between the development of opportunistic and/or serious infections after renal transplantation and circulatory T-cell ageing parameters prior to, and 3 and 6 months after transplantation is analyzed. None of the analyzed T-cell ageing parameters were predictive for the development of infections after transplantation. However, the T-cell kinetics at 3 and 6 months after transplantation rather reflected the exposure to infections. This suggests that the current circulatory T-cell ageing parameters are not predictive for infections after renal transplantation.

In **chapter 5** T-cell ageing parameters within the lymph node are analyzed to assess whether the changes observed in the peripheral blood of end-stage renal disease patients also occur in the lymph nodes. T-cell ageing parameters such as the frequency of recent thymic emigrants, the TREC content and the different subsets of T cells showed significant correlations between the lymph node and the peripheral blood. The effect of age could also be observed within the lymph nodes. A decline in naive T cells and an increase in memory T cells was seen with increasing age. On the other hand, the highly differentiated T cells were close to absent in the lymph node. Next to this, the effect of CMV on T-cell differentiation could not be observed within the lymph node in contrast to the peripheral blood. Thus, T-cell ageing parameters in the lymph node are strongly correlated with the peripheral blood, while the highly differentiated T cells mainly reside within the peripheral blood.

In **chapter 6** the association between T-cell composition of the lymph node and the development of rejection within 3 months after transplantation was analyzed. Patients that developed rejection within three months after transplantation, had a lower frequency of $CD4^+$ T cells and a higher frequency of $CD8^+$ T cells leading to a lower CD4:CD8 ratio within the lymph nodes. Furthermore, there were no functional differences with regard to alloreactivity between T cells from the lymph node and T cells from the peripheral blood.

DISCUSSION

This thesis describes the different aspects of premature T-cell ageing with regard to the predictive value of this ageing process with regard to clinical outcomes and its effect in different compartments of the body. Previous studies have shown that patients with ESRD have a prematurely aged circulatory T-cell immune system (11, 13, 125). It is shown that their circulatory immunological age can be 20 to 30 years older than their calendar age (11). This uremia associated premature ageing process is comparable with the physiological ageing that can be observed in elderly healthy individuals (20, 58).

Furthermore, this premature circulatory T-cell ageing in ESRD patients is irreversible, even after renal transplantation (125).

In the setting of renal transplantation, a highly differentiated T-cell system can lead to prevention of early acute rejection (36). We showed that a high number of $CD4^+CD28^{null}$ T cells prior to renal transplantation is associated with a lower risk for the development of acute rejection within three months after transplantation (36). However, other studies showed that these cells can also be associated with a higher risk for the development of rejection (29, 34). This paradox can be explained by the alloreactive potential of these cells. We hypothesize that these cells have a low alloreactive capacity, which can only be upregulated under optimal conditions, such as the presence of the cytokines IL-15 and IL-21 (article submitted). This could also be the explanation for the improved transplant survival and function seen in patients receiving the co-stimulatory blockade therapy belatacept (106). Blocking of CD80/86 on an antigen presenting cell will prevent this molecule from binding to CD28 on the T cell, which will lead to anergy of the $CD28^+$ T cells (106). This blockade will lead to an overall low risk for alloreactivity when the low alloresponsive profile of $CD4^+CD28^{null}$ T cells is also taken into account. But when $CD86^+$ antigen presenting cells escape such blockades, this could induce the activation of the highly cytotoxic $CD28^{null}$ T cells through the production of IL-15 by for example monocytes and renal tubular epithelial cells. The presence of increased gene expression of IL-15, perforin and granzyme B has been shown in rejecting renal allograft specimens (171, 172). Also, the existence of $CD86^+$ monocytes has been demonstrated in the rejected allograft under belatacept treatment (109). These co-stimulatory blockade resistant rejections are also more severe (92, 108, 109), suggesting the activation of these highly cytotoxic $CD28^{null}$ T cells. Furthermore, in a murine islet transplantation model, an IL-15 antagonist has been shown to reduce $CD8^+$ T-cell infiltration, which was not achieved with co-stimulatory blockade (CTLA4/Fc) therapy alone (173).

Next to this, the low alloreactive capacity of these $CD4^+CD28^{null}$ T cells might be due to their impairment with regard to their T-cell receptor (TCR) diversity. Studies have shown that continuous antigenic stimulation under chronic inflammatory conditions can promote T cells to differentiate into memory T cells with a low TCR diversity (23, 174). Next to this, CMV latency also plays a significant role in the expansion of terminally differentiated T cells (42, 43, 58). These mechanisms together can create a terminally differentiated T-cell pool that is able to recognize only a small group of antigens, which then leads to a reduced ability to recognize new antigens. But when they are able to recognize these antigens, they will require additional stimulation from cytokines such as IL-15 and IL-21 to properly respond and exert possible cytotoxic functions.

In this thesis, the predictive value of T-cell ageing parameters with regard to the development of infections was also investigated. None of these T-cell ageing parameters was able to predict the development of infections after renal transplantation (175). The T-cell kinetics rather represented the consequence of an infectious episode

after renal transplantation (175). This lack of an association between the T-cell ageing parameters and the development of infections might be due to the usage of immunosuppressive drugs which could have influenced these ageing parameters. This might be due to the follow-up period of one year. After this period, the influence of immunosuppression might be less and the effects of an aged immune system might therefore become more prominent.

We also investigated the extent of this premature ageing process in different compartments of the body. Our results showed that the majority of the T-cell ageing parameters were strongly correlated between the peripheral blood and the lymph node (157). However, highly differentiated T cells were virtually absent from the lymph nodes, while the frequency of naive T cells and recent thymic emigrants (RTEs) were higher within the lymph nodes (157). The absence of highly differentiated T cells within the lymph nodes, might be caused by the lack of CCR7 on their surface, impairing them from homing to secondary lymphoid organs. Next to this, the effect of CMV was also not detectable within the lymph nodes, while it has a profound effect on circulatory T cells (157). The absence of highly differentiated T cells and the low frequency of CMV-specific $CD8^+$ T cells within the lymph nodes have also been supported by other studies (145-147). Interestingly the effect of age itself was present within the lymph nodes; frequencies of RTEs and naive T cells declined over increasing age (157). This age-dependent decrease of naive T cells within the lymph nodes have also been observed by other studies (49, 145). The lymph nodes, important for the initiation of immune reactions, could play a role for the development of infections and rejection after transplantation. As a first step to correlate lymph node T-cell composition and functionality with clinical outcomes, we showed that ESRD patients with a high $CD8^+$ T-cell frequency and thus a lower CD4:CD8 ratio within the lymph nodes, were more prone to develop rejection within three months after renal transplantation (in preparation). Our functional analyses showed no differences between the alloreactive capacity of T cells isolated from the lymph node compared to the peripheral blood.

These findings show that immunological ageing can be assessed with the parameters used in this thesis. However, whether these T-cell parameters can be used as predictors for clinical outcomes requires more in depth analysis. For true personalized medicine, a better immunological model is essential, which incorporates the different aspects of this process. For example, molecular mechanisms that are involved in immunosenescence can be a part of this model. Studies have shown that CD28 gene expression declines with increasing age (176, 177). Furthermore, raised levels of transcription factors that are associated with increased cytotoxicity, such as T-bet and eomesodermin were also observed in $CD8^+CD28^{null}$ memory T cells (177). This can be accompanied by investigating microRNA regulation. It has been shown that miR-181a declined with increasing age, leading to an impaired TCR sensitivity in $CD4^+$ T cells (178). Furthermore, other cells of the immune system, such as B cells, are also affected by ageing (179). This means that research regarding interaction between different cells of the immune system will give a better reflection of the true extent of this ageing process. How all these mechanisms together contribute to premature ageing in ESRD is unknown, but needs to be elucidated for a proper model which defines appropriate parameters that can be used for personalized medicine.

Our results show the relationship between T-cell ageing parameters and clinical outcomes as well as the extensiveness of uremia associated T-cell ageing throughout the different compartments of the body. These studies demonstrate that these ageing parameters are not equally contributing to the prediction of different clinical outcomes. Next to that, the lymph nodes seem to be have a different T-cell composition, while age itself does have an effect on this configuration.

CONCLUSION

This research describes the relationship between premature T-cell ageing in patients with end-stage renal disease and the development of rejection and infections after renal transplantation. A highly differentiated T-cell system is protective for the development of rejection after transplantation, probably due to the low alloresponsive character of these cells. On the other hand, T-cell ageing parameters are not a tool to predict the development of infections within one year after transplantation. Next to that, the range of this premature ageing process throughout the body is also evaluated. Although T-cell ageing parameters were strongly correlated between the peripheral blood and lymph nodes, highly differentiated T cells were virtually absent from the lymph nodes.

For a complete understanding of the ageing process in ESRD patients, further research is needed with regard to the interaction between the different cells of the immune system, combined with the molecular changes that occur during this ageing process. This research should include studies integrating these parameters and associate them with clinical outcomes such as rejection and infections. Furthermore, a more in depth analysis of functional impairments of the different immune cells after allogeneic challenge should be performed. These impairments can be at the antigen presentation level and/or at the level of antibody production by B cells. These comparisons can be made by studying the immune cells from an ESRD patient and cells from a healthy individual. The clinically relevant parameters could then be transformed to an algorithm. This algorithm can be used as a more proper tool for a more solid risk assessment with regard to clinical outcomes. This then will provide a more personalized approach for the use of immunosuppression. Thus, this thesis describes only the T-cell aspect of the affected immune system in end-stage renal disease patients.

Chapter 8

DUTCH SUMMARY
(NEDERLANDSE SAMENVATTING)

NEDERLANDSE SAMENVATTING

De nieren spelen een belangrijke rol bij een aantal essentiële processen in het lichaam. Ze zorgen voor een balans in de osmolaliteit en het volume van de extracellulaire vloeistof. Dit resultaat wordt bereikt door het uitscheiden van water, elektrolyten en afvalstoffen. Daarnaast zijn de nieren betrokken bij de productie van een aantal hormonen zoals erytropoëtine (productie van rode bloedcellen), calcitriol/actief vitamine D (botmetabolisme) en renine (vaatvernauwing en verhoging van de bloeddruk). Wanneer de nieren hun functie verliezen, zal dit dus effect hebben op alle voorgaande processen.

Naast al het bovengenoemde, heeft het verlies van nierfunctie ook invloed op het afweersysteem. Eerder is aangetoond dat deze patiënten een verouderd T-cel afweersysteem hebben. De immunologische leeftijd van deze patiënten kan 20-30 jaar ouder zijn dan hun kalenderleeftijd. Deze versnelde veroudering wordt waarschijnlijk geïnitieerd door de aanwezige uremie, die een pro-inflammatoir milieu creëert. Dit milieu zal tot een continue activatie leiden van het immuunsysteem, waaronder dus ook de T-cellen. Dit proces kan mede ten grondslag liggen aan het feit dat patiënten met eindstadium nierfalen minder reageren op vaccinaties en een verhoogde kans hebben op infecties. Dit betekent dat deze veranderingen in het afweersysteem ook een rol zullen spelen bij niertransplantatie. Een transplantatie is de beste optie in het kader van nierfunctie vervangende therapie, maar aan de andere kant brengt een dergelijke ingreep het gebruik van afweer onderdrukkende middelen met zich mee. Deze middelen verminderen weliswaar de kans op afstoting na transplantatie, maar verhogen aan de andere kant de kans op infecties. Bij patiënten met eindstadium nierfalen, waarvan het afweersysteem al is aangedaan door nierfunctie verlies, zal het gebruik van afweer onderdrukkende middelen dus een ernstiger effect kunnen hebben. Dit maakt het definiëren van immunologisch voorspellende parameters voor het ontwikkelen van afstoting en infecties na transplantatie noodzakelijk, om een zo individueel mogelijke benadering voor afweer onderdrukkende therapiekeuze te kunnen bewerkstelligen.

In **hoofdstuk 1** wordt de versnelde veroudering van het T-cel afweersysteem bij patiënten met eindstadium nierfalen en de verschillende T-cel parameters die bepaald kunnen worden uit bloed en lymfeklieren uiteengezet. De parameters die gebruikt kunnen worden, zijn: frequentie van naïeve T-cellen die afkomstig zijn uit de thymus, de differentiatiestatus van de T-cellen en de telomeerlengte van de T-cellen.

In **hoofdstuk 2** worden de verschillende T-cel parameters vanuit het bloed voorafgaand aan transplantatie in relatie gebracht met het risico op afstoting kort (tot 3 maanden) na niertransplantatie. Hieruit bleek dat een hoog aantal $CD4^{+}CD28^{nul}$ T-cellen met name voorkwam bij patiënten zonder afstoting na niertransplantatie. Een vergelijkbare trend werd ook waargenomen voor de $CD8^{+}CD28^{nul}$ T-cellen. De toegenomen aanwezigheid van deze doorgedifferentieerde cellen is dus geassocieerd met een lager risico op vroege afstoting na niertransplantatie.

In **hoofdstuk 3** wordt verder ingegaan op de $CD4^{+}CD28^{nul}$ T-cellen met betrekking tot hun alloreactief potentieel. Hierbij is onderzocht hoe deze cellen reageren op allogene stimulatie en wat de invloed is van exogene cytokines. Hieruit kwam naar voren dat zonder cytokines, de $CD4^{+}CD28^{nul}$ T-cellen niet in staat waren tot proliferatie, degranulatie, cytotoxicitieit of cytokine productie na allogene stimulatie. Toevoegen van IL-15 (met/zonder IL-21) verhoogde de alloreactiviteit. Deze bevindingen laten dus zien, dat het milieu waar deze cellen zich in bevinden van invloed is op hun alloreactief potentieel.

In **hoofdstuk 4** worden de verschillende T-cel parameters vanuit het bloed voorafgaand, en 3 en 6 maanden na transplantatie in relatie gebracht met het ontwikkelen van opportunistische en/of serieuze infecties. Hieruit bleek dat geen van de onderzochte T-cel parameters een voorspellende waarde had voor het optreden van infecties na niertransplantatie. De kinetiek van de parameters na transplantatie was een weerspiegeling van de opgetreden infecties. Dit suggereert dat de huidige T-cel parameters niet geschikt zijn voor het voorspellen van infecties na transplantatie.

In **hoofdstuk 5** wordt verder onderzocht of de waargenomen T-cel veranderingen in het bloed ook kunnen worden waargenomen in de lymfeklieren. Hieruit kwam naar voren dat de verschillende parameters tussen de twee compartimenten sterk aan elkaar waren gecorreleerd. Daarnaast werd er een effect van veroudering gezien in de lymfeklieren, waarbij een afname van naïeve T-cellen en een toename van geheugen T-cellen werden waargenomen. Daartegenover werd geconstateerd dat de doorgedifferentieerde cellen in hoge frequenties aanwezig waren in het bloed, terwijl deze cellen nagenoeg afwezig waren in de lymfeklier. Hiernaast werden ook de effecten van CMV niet waargenomen in de lymfeklier, terwijl deze wel duidelijk te observeren waren in het bloed van dezelfde patiënt. Hieruit kan geconcludeerd worden dat de T-cel parameters in bloed en lymfeklier sterk met elkaar zijn gecorreleerd, maar dat de doorgedifferentieerde cellen zich voornamelijk in het bloed bevinden.

In **hoofdstuk 6** wordt verder ingegaan op de functionele eigenschappen van de cellen geïsoleerd uit de lymfklier vergeleken met de cellen verkregen uit het bloed. In dit hoofdstuk werd geobserveerd dat een lagere frequentie aan $CD4^{+}$ T-cellen en een hogere frequentie aan $CD8^{+}$ T-cellen, dus een lagere CD4:CD8 ratio in de lymfeklier, geassocieerd was met het ontwikkelen van rejectie binnen 3 maanden na transplantatie. Daarnaast werd geobserveerd dat er functioneel geen verschillen waren m.b.t. alloreactiviteit tussen T-cellen vanuit de lymfeklier en T-cellen vanuit het perifere bloed.

Concluderend laten de resultaten in dit proefschrift zien dat een meer gedifferentieerd T-cel immuunsysteem wellicht een gunstiger prognose biedt in het kader van afstoting na niertransplantatie. Hierbij gaat het voornamelijk om een relatie met de aanwezigheid van veel $CD4^{+}CD28^{nul}$ T-cellen. Deze cellen lijken beperkt in hun alloreactiviteit, maar of deze cellen de bepalende factor zijn in het voorkomen van rejectie is nog niet met zekerheid te zeggen. Hoogstwaarschijnlijk maken deze cellen meer een deel uit van een aangedaan immuunsysteem, dat minder in staat is om nieuwe antigenen te herkennen en hierop adequaat te reageren. Daarnaast zijn er belangrijke intra-individuele verschillen, omdat niet alle compartimenten in het lichaam even sterk zijn aangedaan door nierfunctieverlies. Hieruit volgt logischerwijs dat er meer diepgaand onderzoek nodig is op het gebied van veroudering bij patiënten met eindstadium nierfalen. In de bovenstaande onderzoeken lag de nadruk op de T-cellen. In vervolgonderzoeken zou de focus meer moeten liggen op de interactie van T-cellen met de B-cellen en/of de antigeen presenterende cellen in de verschillende compartimenten van het lichaam. Voor een completer beeld, is onderzoek op moleculair niveau, zoals transcriptiefactoren en micro-RNA, noodzakelijk, waarbij epigenetica een belangrijke rol kan spelen om in te grijpen in deze processen.

Chapter 9

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REFERENCES

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APPENDICES

CURRICULUM VITAE

Burç Dedeoğlu was born on December the 11th 1989 in Roosendaal, the Netherlands. From 2002 to 2008 he attended the VWO at Thorbecke College in Rotterdam, the Netherlands. In September 2008 he started his Medicine Study at the Erasmus University Rotterdam and earned his medical degree in August 2014. In October 2014 he started with his PhD project at the Transplantation Laboratory of the Internal Medicine Department, Section Nephrology and Transplantation, at the Erasmus Medical Center, under the supervision of prof. dr. Carla Baan, dr. Michiel Betjes and dr. Nicolle Litjens. This thesis is a representation of this PhD project.

LIST OF PUBLICATIONS

Dedeoglu B., de Geus H.R., Fortrie G., Betjes M.G.H.; Novel biomarkers for the prediction of acute kidney injury in patients undergoing liver transplantation. *Biomarkers in Medicine, 2013 Dec;7(6):947-57*

Dedeoglu B.*, Meijers R.W.*, Klepper M., Hesselink D.A., Baan C.C., Litjens N.H.R., Betjes M.G.H.; Loss of CD28 on Peripheral T Cells Decreases the Risk for Early Acute Rejection after Kidney Transplantation. *PLoS One, 2016 Mar 7;11(3):e0150826*
*Both authors equally share first authorship

Dedeoglu B., Meijers R.W., Klepper M., Hesselink D.A., Baan C.C., Litjens N.H.R., Betjes M.G.H.; Uremia-Associated Premature Ageing of T Cells Does Not Predict Infectious Complications After Renal Transplantation. *American Journal of Transplantation, 2016 Aug;16(8):2324-33*

Dedeoglu B., de Weerd A.E., Huang L., Langerak A.W., Dor F.J., Klepper M., Verschoor W., Reijerkerk D., Baan C.C., Litjens N.H.R., Betjes M.G.H.; Lymph node and circulating T cell characteristics are strongly correlated in end-stage renal disease patients, but highly differentiated T cells reside within the circulation. *Clinical and Experimental Immunology, 2017 May;188(2):299-310*

Dedeoglu B., Litjens N.H.R., Klepper M., Kraaijeveld R., Verschoor W., Baan C.C., Betjes M.G.H.; CD4^{+}CD28^{null} T Cells Are Not Alloreactive Unless Stimulated by IL-15. *American Journal of Transplantation 2017 August; accepted for publication*

Litjens N.H.R., Huang L., **Dedeoglu B.**, Meijers R.W.J., Kwekkeboom J., Betjes M.G.H.; Protective CMV-Specific T-Cell Immunity Is Frequent In Kidney Transplant Patients Without Serum Anti-CMV Antibodies. *Frontiers in Immunology 2017 August; accepted for publication*

MANUSCRIPTS SUBMITTED OR UNDER REVIEW

Dedeoglu B., Litjens N.H.R., Klepper M., Reijerkerk D., Baan C.C., Betjes M.G.H.; T-Cell Composition of the Lymph Node Is Associated with the Risk for Early Rejection after Renal Transplantation. *Submitted to Frontiers in Immunology, 2017 August*

PHD PORTFOLIO

Name PhD student:	Burç Dedeoğlu
Erasmus MC Department:	Internal Medicine, section Nephrology and Transplantation
Research School:	Postgraduate School Molecular Medicine
PhD period:	October 2014 – September 2017
Promotor:	Prof. dr. Carla C. Baan
Copromotors:	Dr. Michiel G.H. Betjes and Dr. Nicolle H.R. Litjens

General courses		
Advanced Immunology	2015	
Biomedical English Writing and Communication	2015	
Biostatistical Methods I: Basic Principles	2015	
Research Integrity	2015	
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2016	
(Inter)national conferences		
'Wetenschapsdagen', Dpt. of Internal Medicine Erasmus MC, Antwerp, Belgium	2015	Attended
Dutch Nephrology Days, Dutch Society for Nephrology (NND), Veldhoven, the Netherlands	2015	Poster
European Society for Organ Transplantation, Brussels, Belgium	2015	Brief presentation
CMV and Immunosenescence, Amsterdam, the Netherlands	2015	Attended
Wetenschapsdagen, Dpt. of Internal Medicine Erasmus MC, Antwerp, Belgium	2016	Presentation
'Bootcongres', Dutch Transplantation Society (NTV), Groningen, The Netherlands	2016	Presentations (2x)
Dutch Nephrology Days, Dutch Society for Nephrology (NND), Veldhoven, the Netherlands	2016	Presentation
American Transplant Congress, Boston, USA	2016	Posters (2x)
British Society for Immunology (BSI) & Dutch Society for Immunology (NVVI) joint meeting, Liverpool, UK	2016	Posters (2x)
'Wetenschapsdagen', Dpt. of Internal Medicine Erasmus MC, Antwerp, Belgium	2017	Poster
'Bootcongres', Dutch Transplantation Society (NTV), Groningen, The Netherlands	2017	Poster
Dutch Nephrology Days, Dutch Society for Nephrology (NND), Veldhoven, the Netherlands	2017	Brief presentation Poster
American Transplant Congress, Chicago, USA	2017	Posters (2x)
European Society for Organ Transplantation, Barcelona, Spain	2017	Presentation Poster

PhD Portfolio. (continued)

Teaching activities	
Supervising, teaching and grading an MLO student during his final (9 months) internship at the department	2015-2016
Supervising and teaching two high school (VWO) students during their 'Junior Med School' programme	2017
Membership	
Dutch Transplant Society (Nederlandse Transplantatie Vereniging, NTV)	

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Got you there. Wanneer we samen experimenten moesten doen, wist ik zeker dat er gelachen zou worden. Bedankt voor al je hulp tijdens de sort experimenten en je goede/droge gevoel voor humor.

Wenda, ook jij hebt me veel geholpen met de sort experimenten. Daarnaast hield je ook altijd goed bij hoeveel dagen ik nog vrij had staan (die 'waarschuwingsmails' zullen zeker niet vergeten worden). Bedankt voor al je hulp.

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